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Tese orientada pelo Doutor Henrique Veiga-Fernandes e pela

Professora Doutora Margarida Telhada

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I. RESUMO

A mucosa intestinal abriga uma vasta comunidade de microorganismos que contribuem para o normal funcionamento dos organismos superiores. Encontra-se dotada de um sistema imunitário que se compartimentaliza em diferentes estruturas linfóides que permitem a interacção com o meio exterior. O sistema imune intestinal contribui para a homeostase intestinal, tolerando os organismos comensais e defininindo respostas inflamatórias contra patogénios. Num contexto homeostáctico, o estabelecimento de mecanismos que regulam o desenvolvimento de respostas imunes após interacção com um organismo ou antigénio desconhecido tornam-se indispensáveis. A influência de nutrientes no estabelecimento e regulação destas respostas tem vindo a ser demostrada nos últimos anos. Contudo, pouco ainda é conhecido sobre os mecanismos moleculares subjacentes à interacção entre nutrientes e as células imunes.

A deficiência em vitamina A afecta aproximadamente 250 milhões de pessoas em todo o mundo, sendo responsável por um grave aumento de morbidez a infecções gastrointestinais e respiratórias. O ácido retinóico (RA) é um metabolito da vitamina A que desempenha importantes funções no desenvolvimento do sistema imunitário e na regulação das respostas imunes. Uma resposta imunitária adequada requer a existência de mecanismos moleculares que permitam o rápido recrutamento de células imunes para combater o alastramento de uma infecção. Entre estes mecanismos inclui-se a aquisição da expressão de moléculas designadas por marcadores de residência que permitem o reconhecimento de sinais quimiotácticos e a retenção das células num determinado tecido.

O receptor CCR9 e as integrinas $\alpha_4\beta_7$ são os marcadores de residência mais importantes para o estabelecimento de células T na mucosa intestinal. Estudos anteriores relatam que o processo de aquisição da expressão de CCR9 e $\alpha_4\beta_7$ é promovido por células dendríticas (DCs) $CD103^{pos}$ provenientes do intestino que metabolizam a vitamina A e fornecem RA a células T na periferia. Nas células T, o RA interage com receptores do ácido retinóico (RAR) desencadeando vias de sinalização que induzem a expressão de CCR9 e de $\alpha_4\beta_7$. Os linfócitos intra-epiteliais (IELs) residem no epitélio e constituem a primeira linha de combate do sistema imunitário nas mucosas. Estes linfócitos caratecterizam-se em duas classes distintas: IELs não convencionais (ou naturais) - linfócitos T γδ e T αβ que expressam o homodímero CD8αα, e células T γδ que não expressam CD8 - e convencionais (ou induzidas) - compostas pelas células T $CD4^{pos}$ e $CD8^{pos}$ clássicas.

Apesar da função do RA no recrutamento e estabelecimento de células T na *lamina propria* intestinal já ter sido demonstrada, pouco é sabido sobre o seu papel na função e migração dos IELs, como também sobre a própria dinâmica migracional destas células. Mais ainda, estas experiências foram realizadas através de dietas pobres em vitamina A, depletando o RA de forma promíscua. Por esta razão propusémo-nos a estudar o papel do RA na função e estabelecimento de células T no epitélio intestinal. Para tal, usámos ratinhos que possuem uma forma do RARα truncada após o aminoácido 403 (*RARaT403*) inserida no gene ubiquamente expresso *ROSA-26*, com um codão *stop* flanqueado por sequências *loxP* (ratinhos T403). O *RARaT403* é incapaz de iniciar a cascata de sinalização a jusante do RARα e possui dominância negativa para com todas as isoformas do RAR. De forma a bloquear a sinalização do RA em células T, cruzámos estes ratinhos com outros que possuem um recombinase *Cre*, que reconhece sequências *loxP*, sob controlo do promotor do gene *CD2* humano (ratinhos *CD2cre T403*).

Os nossos resultados demonstram que uma deficiente sinalização de RA resulta numa forte diminuição do número de células T γδ, e de células T αβ CD8αα e CD8 αβ no epitélio, utilizando ratinhos com a expressão do RARaT403 em heterozigotia (*CD2cre T403*^{het}). Observámos também uma diminuição de células T γδ, células T αβ e células inatas linfóides (ILCs) na *lamina propria* intestinal.

A análise de timócitos durante o fluxo migratório de células T para o intestino, decorrente nas primeiras três semanas de vida do ratinho, apresentou uma acumulação de células T CD8^{pos} no timo e diminuição de IELs não-convencionais no intestino. De forma a determinar se a diminuição observada seria causada por uma deficiente migração, baixa proliferação, ou por uma manutenção inefectiva, avaliámos o ciclo celular dos IELs e analisámos a expressão de CCR9 e de factores envolvidos na sobrevivência dos IELs em ratinhos *CD2cre T403*. Considerámos que o RA medeia o processo de migração de células T para o epitélio, atendendo à diminuição dos níveis de transcrito *Ccr9* e à semelhante perfil de incorporação de BrdU em todas as populações de IELs de ratinhos *CD2cre T403*. Em reforço desta hipótese, os níveis de transcrito *Ahr* - fundamental para a sobrevivência das IELs – encontravam-se normais, e os níveis de *Bcl2* e *Bcl2l1* – proteínas anti-apoptóticas – estavam semelhantes ou sobreexpressos em ratinhos *CD2cre T403*.

De forma a compreender os mecanismos que regem a aquisição de tropismo em timócitos, determinámos a janela de tempo de desenvolvimento de células T em que o RA promove a aquisição de tropismo intestinal, recorrendo a ratinhos *Rorc-cre T403*^{het}. Os nossos resultados

sugerem que apenas em células T αβ não-convencionais, o tropismo é induzido após a fase DP (dupla positiva – timócitos que expressam CD8 e CD4). Deste modo, o nosso trabalho revela que a aquisição de tropismo em todos os IELs $CD8^{pos}$ deverá ocorrer no timo, e que o RA é imprescindível neste processo.

Para além do papel do RA no recrutamento de células T para a periferia, demonstrámos também que o RA participa em processos de imunomodelação de IELs não-convencionais. Concretamente, a análise do perfil de citocinas de ratinhos com deficiente sinalização retinóide apresentou um aumento da percentagem de células produtoras de IL-17 em IELs γδ e αβ não-convencionais comparativamente a ratinhos controlo. Por conseguinte, sugerimos que o RA poderá estar envolvido em mecanismos de regulação de respostas do tipo Th17 no epitélio.

Por último, demonstrámos que o RA é crucial para a homeostasia de ILC3s na *lamina propria* intestinal. A análise de ratinhos *CD2cre T403*^{homo} revelou uma diminuição dos números de ILC3s no intestino. Visto não terem sido encontradas diferenças em ratinhos *CD2cre T403*^{het}, considerámos que o fenótipo depende da dose de RA. A análise de ratinhos *Rorc-cre T403*^{homo}, onde a dominância negativa do mutante T403 é adquirida aquando a diferenciação das ILCs em ILC3s, apresentou uma dimuição das últimas. Por outro lado, a expressão de genes fundamentais para a sobrevivência das ILCs, bem como o ciclo celular e o número de ILCs em apoptose demonstraram ser semelhantes na ausência de sinalização retinóide. Neste sentido, sugerimos que o RA é fundamental para a diferenciação das ILCs, apesar de não contribuir para a sobrevivência e expansão após o seu desenvolvimento. Em contrapartida observámos que, nos mesmos ratinhos, os níveis de transcrito *Rorc*, que codifica para a proteína RORγt, se encontrava reduzido em ILC3s. Neste sentido, o nosso trabalho demonstra a importância do RA na manutenção da expressão de RORγt, principal regulador da diferenciação de ILCs em ILC3s.

Em suma, os nossos resultados sugerem uma função imprescindível do ácido retinóico na regulação do sistema imunitário do intestino, em particular no *imprinting* de tropismo intestinal em células T não-convencionais no timo, e no desenvolvimento das ILCs.

Palavras-chave: Ácido retinóico, tropismo, migração, intestino, sistema imunitário do intestino, linfócitos intra-epiteliais, células inatas linfóides.

II. SUMMARY

Intraepithelial T lymphocytes (IELs) are key components of mucosal immunity, defining a front-line of defence against invasive microorganisms. However, the molecular mechanisms controlling IEL function and maintenance are still poorly defined. Retinoic acid (RA), a vitamin A metabolite, has been shown to mediate important processes in the immune system. Herein, we aimed to determine the role of retinoic acid signalling in the regulation of IEL development and establishment in the intestine. Remarkably, mice with deficient RA signalling in T cells exhibited significantly impaired *lamina propria* lymphocytes (LPL) and IEL pools. This was associated with the expression deficit of the gut-homing marker CCR9 in all IEL subsets while gene expression of a master regulator of IEL maintenance, *Ahr,* and their proliferative capacity was unaffected. Strikingly, monitoring of thymocytes during early gut colonization revealed that suboptimal RA signalling results in reduction of T cells in the gut and normal or increased T cells in the thymus. Moreover, we found that impairment of RA signalling at different thymic developmental stages impacts IEL subsets differently. Notably, we found evidence that RA signalling affects group 3 innate lymphoid cells (ILC3s) in a dose dependent manner, although it does not affect their maintenance or expansion. We also found that RA regulates *Rorc* gene expression and RORγt protein levels in ILC3s. Our findings illustrate that RA is an essential dietary component for normal development and function of the enteric immune system and unravel a putative function in thymic imprinting of gut-tropism to unconventional IELs.

Key words: Retinoic acid, gut-tropism, migration, gut, enteric immune system, intraepithelial lymphocytes, innate lymphoid cells.

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IV. INTRODUCTION

1. GENERAL ASPECTS

Evolution endowed organisms with a specialized immune system capable of performing prompt immune responses against foreign aggressions. The cells that constitute this system arise from a biological differentiation process named haematopoiesis [5, 6]. After their generation, immune cells migrate from primary lymphoid organs (PLOs) to secondary lymphoid organs (SLOs) [7, 8, 9], which constitute critical interfaces of physical and immunological interaction with foreign products, such as bacteria and fungi [10, 11]. Secondary lymphoid organs are well-organized lymphoid structures anatomically located in discrete regions of the mammalian body [7, 12]. These structures include the spleen, lymph nodes, tonsils and mucosal-associated lymphoid tissues (MALT) [7, 9].

T and B lymphocytes are critical arms of adaptive immunity. Naïve lymphocytes with a restricted specificity to a given antigen are extremely rare – in the order of 1 in 10^6 – thus initiation of primary immune responses would be very inefficient in the wide context of peripheral body tissues [13]. SLOs define congregation sites for naïve lymphocytes that arrive from primary lymphoid organs such as the thymus and the bone marrow, and for antigen-bearing antigen-presenting cells (APCs) coming from the periphery [14]. They are connected through lymphatic vessels and blood stream, allowing continuous circulation of immune cells between SLOs [12, 15]. Thus, these structures increase the probability of a naïve cell to encounter its complementary epitope presented by an APC [14]. SLOs also maximize the efficiency of immunosurveillance, as naïve cells undergo proliferative expansion that leads them to an activated state, after interacting with its complementary epitope [14 16].

The great majority of immune cells reside within the mucosal tissues (MALT), which are the most extensive components of the immune system [9, 17]. In the intestine, these lymphoid structures are named GALT (gut-associated lymphoid tissues) [9, 17], that heavily contribute for host defense [18].

2. GUT-ASSOCIATED LYMPHOID TISSUE (GALT)

The mammalian gastrointestinal tract (GI) is continuously exposed to external material, ranging from food antigens and commensal bacteria to putative pathogens [2, 4]. The luminal duct is an extensive surface area harbouring an ecosystem that can reach 10^{14} bacteria [19]. Thus, balance between maintenance of tolerogenic responses and the development of immunogenic protection is essential for intestinal homeostasis [11, 20]. This commitment is accomplished by the enteric immune system, constituted by the GALT and the diffuse cells within the *lamina propria* (LP) and epithelium [21]. The GALT is a network of secondary lymphoid structures including mesenteric lymph nodes (mLNs), Peyer's patches (PPs), cryptopatches (CPs) and isolated lymphoid follicles (ILFs) (Figure 1A) [21]. All these lymphoid structures are fundamental for host defence through the establishment of immunological barriers and constitute critical interfaces of interaction with the foreign microorganisms [2, 11].

2.1. Mesenteric lymph nodes

Lymph nodes (LNs) are strategically distributed throughout the body, ensuring efficient antigen (Ag) scanning of the draining lymph from distinct anatomical tissues [22]. Structurally, LNs are compartmentalized into distinct B cell and T cell areas [23]. After sensitization peripheral Ag-loaded dendritic cells (DCs) congregate in T cell areas allowing the encounter of Ag-specific T cells (Figure 1A) [22, 23]. Therein, antigen presentation permits the establishment of efficient adaptive immune responses [9, 23, 24].

Mesenteric lymph nodes (MLNs) lie between the mesentery membranes, in the lower abdominal cavity [25]. They constitute amplification sites of antigen-specific responses initiated in the gut in order to prevent the systemic spread of infections [22]. Although infection clearance requires rapid migration of activated T cells to the site of infection, MLNs are not vital for the generation of gut-tropic T cells since mice without MLNs still generate T cells homing to the gut [26] – MLNs may constitute inductive sites for tolerance towards food and commensal Ags [27]. They form the border between enteric immunity and the remainder of immune system, providing anatomical and immunological containment of invasive microbiota [28].

2.2. Peyer's Patches

First identified by the pathologist Johann Conrad Peyer in 1966, PPs constitute the largest specialized lymphoid structures in the small intestine [29, 30, 31]. In laboratory mouse strains, the average intestine has 8-12 PPs [32] located at the anti-mesenteric wall of the gut [33]. PPs structure is defined by the presence of several B cell follicles with in-between T cell areas [34]. Similarly to LNs, high endothelial venules (HEV) in PPs are surrounded by T cell areas, and enable the trafficking of naïve lymphocytes into the PPs [35].

The luminal side of PPs reveal a specialized epithelial layer called follicle-associated epithelium (FAE) lacking crypts or villi [31]. FAE display a specialized epithelial cell, named microfold cell (or M cell), that enables sampling of luminal Ags and intact organisms to the PP environment [31]. In the *lamina propria* side, the transported Ags encounter clustered cells such as DCs, $CD4^{pos}$T cells and B cells that define the subepithelial dome (SED) of PPs [31]. This region allows the initiation of antigen processing and presentation by DCs in PPs that will define particular immune responses [36]. Following antigenic stimulation, follicular B cells may form germinal centres where they undergo class switching recombination from IgM to IgA (Figure 1A) [31].

2.3. Cryptopatches and isolated lymphoid follicles

SLOs organogenesis occurs mostly during embryogenesis, while other lymphoid structures assemble after birth. Cryptopatches are small clusters of $ROR\gamma t^{pos}$ innate lymphoid cells (Group 3 ILCs) surrounded by DCs that also contain T cells, common lymphoid progenitors (Lin^{neg} c-kit^{pos} IL-$7R\alpha^{pos}$) and few or none B cells [10, 21, 24, 37, 38]. These lymphoid aggregates only begin to develop below crypts of murine's intestine around postnatal day 14 [39].

CPs have been postulated to function as maturation centres of intraepithelial lymphocytes (IELs) [40]. This was hypothesized after observations that T cells could be reconstituted in the intestine with transfer assays of CP cells into lymphopenic mice [40]. In contrast, RORγt is expressed in all DP thymocytes, and genetic fate mapping experiments suggested that IELs generate from a $ROR\gamma t^{pos}$ progenitor [21]. Additionally, in the absence of CPs and other lymphoid structures named isolated lymphoid follicles (ILFs), IELs were present in normal numbers [11, 38]. Moreover, no evidence of recombinase-activating gene (*Rag*) activity was found in CPs [41]. These results suggest that cyptopatches are not required for IEL generation [11].

Group 3 innate lymphoid cells (group 3 ILCs) are critical initiators of CP formation (Figure 1A). RORγt-deficient mice – in which group 3 ILCs are absent – lack CPs and ILFs [21]. In addition, *Ahr* knockout mice, that display impaired postnatal development of group 3 ILCs, fail to develop CPs and ILFs [42, 43].

In fact, CPs are the anlagen of ILFs [44]. ILF development occurs after B cell recruitment mediated by cryptopatch-resident ILC3s [11]. ILFs are relatively large lymphoid structures composed by a single B cell follicle surrounded by RORγt^{pos} ILCs [11]. In resemblance to PPs, follicular B cells also become plasma cells within ILFs, producing IgA important for luminal microbiota control [44]. The similarities with PPs are further underlined by the presence of germinal centres, and M cells [11, 45, 46]. ILFs are therefore important interfaces for the development of IgA-mediated responses (Figure 1A).

Contrarily to CPs, in which formation is developmentally programmed, ILFs maturation depends on microflora stimuli [17, 37]. Microbe-associated molecular patterns (MAMPs) induce ILFs assembly via NOD-1-dependent recognition by epithelial cells (ECs) [47]. Additionaly, germ-free mice display impaired ILF development while CPs are present in normal numbers [47]. In accordance to these observations, ILFs are only noticeable around weaning time, when bacterial load is increased with solid food intake, and their formation is thought to be continuous throughout life [47].

Lastly, other factors such as $LT\alpha_1\beta_2$ and CXCL13 are involved in ILFs development. $LT\alpha_1\beta_2$ which is expressed by ILCs binds to LTβR on stromal cells[48], up-regulating the expression of chemokines, as CXCL13, and adhesion molecules in order to recruit and retain lymphocytes [17, 49]. Further evidence was provided by LTα-deficient and in LTβ-deficient mice, where both CPs and ILFs are absent [50].

All these data suggests a reversible nature of ILFs maturation that defines a mechanism of immune homeostasis within the gut [29, 51, 52].

3. EPITHELIAL CELL BARRIER

The colonization of the intestinal mucosal tissues begins at birth, when the new-born is exposed to mother's microbiota [4]. Hereupon, the gastrointestinal tract becomes the habitat of multiple distinct microbes that coexist with the host in a mutually beneficial relationship [4]. The crosstalk between intestinal microbiota, epithelial cells (ECs) and immune cells promotes the settlement of protective responses against pathogens [53]. On the other hand, chemical and physical mechanisms such as antimicrobial peptide (AMP) secretion, mucus production, and intestinal microenvironment sampling at the epithelium permit the maintenance of enteric homeostasis [54]. Importantly, failure of these processes often correlates with susceptibility to infections and intestinal pathologies [53, 54, 55].

3.1. Epithelial cells

The intestinal epithelium is an extensive surface that undergoes constant self-renewal [56]. Epithelial cell replenishment occurs by differentiation of cryptic stem cells and exfoliation of senescent epithelial cells located on the tips of the villi [53]. This process is performed without disruption of tight junctions, enabling the maintenance of epithelial barrier integrity and thus avoiding the dissemination of microorganisms [53]. Microbial sensing by epithelial cells occurs mainly through the interaction with pattern recognition receptors (PRR) that recognize MAMPs [57, 58]. Toll-like receptors (TLRs) and cytosolic NOD-like receptors (NLRs) trigger signalling pathways that not only induce production and release of AMPs, but also promote the turnover of ECs and strengthen the cell junctions within the epithelial wall [53, 57, 58]. Activation of transcription factor NF-κB, and the immune cytokine and chemokine IL-13 and CXCL10 control epithelial cell turnover hence regulating their continuous replacement [53, 54]. Nevertheless, aberrant or sustained PRR signalling events can lead to chronic intestinal pathologies like the intestinal inflammatory diseases (IBD) [55, 57, 58].

3.2. Paneth cells and antimicrobial peptides

Paneth cells are specialized enterocytes that proficiently produce molecules that prevent bacterial penetration and expansion [54]. Among those are small, cationic peptides like α-defensins and C-type lectins called AMPs (Figure 1B) [59, 60]. Beyond their antimicrobial function,

AMPs also act as opsonins and chemoattractants, enhancing pathogen encounter by immune cells [59]. Production of α-defensins causes permeabilization of bacterial membranes and also recruits neutrophils, monocytes and lymphocytes to the site of infection [60]. Defensins also contribute to resolution of inflammation by blocking IL-1β (a cytokine that promotes inflammatory responses) in activated monocytes [60]. Furthermore, secretion of C-type lectins such as RegIII proteins causes lysis of gram^{pos} bacteria through direct binding to bacterial peptidoglycans [59].

Cytokines also take part in the promotion of AMPs production by Paneth cells. Upon dissemination of luminal bacteria, IL-22 signalling increases AMPs release (Figure 1B). In dextran sodium sulphate–induced colitis (DSS is a glucose polymer used to induce colitis in mice), administration of IL-22 ameliorates disease severity and re-establishes goblet cells and mucus layer production. Therefore, IL-22 has emerged as a key element in epithelial barrier conservation and homeostatic responses towards the microflora [34, 52, 61, 62, 63].

3.3. Goblet cells and mucus layer

Goblet cells are epithelial cells that set up the mucus layer through the production of glycoproteins named mucins [33] that are embedded in the intestinal epithelial wall by the glycocalyx coat [64]. Mucins are the main components of mucus layer and MUC2 is the most abundant one in the small intestine [55]. However, mucin misassembly, disruption of MUC2 production or goblet cell loss are commonly observed in intestinal diseases like IBDs [54, 55].

The mucus layer is composed of two distinct sub-layers: an internal thin layer that is sterile and an external thick layer that contains bacteria [4]. Mucin assembly in the internal layer provides a gel-like physical barrier that impedes microorganisms' accessibility to the epithelium [54, 55, 58], and its viscosity allows embedding and retention of AMPs and IgAs that ensure efficient protection against invasive bacteria (Figure 1B) [55].

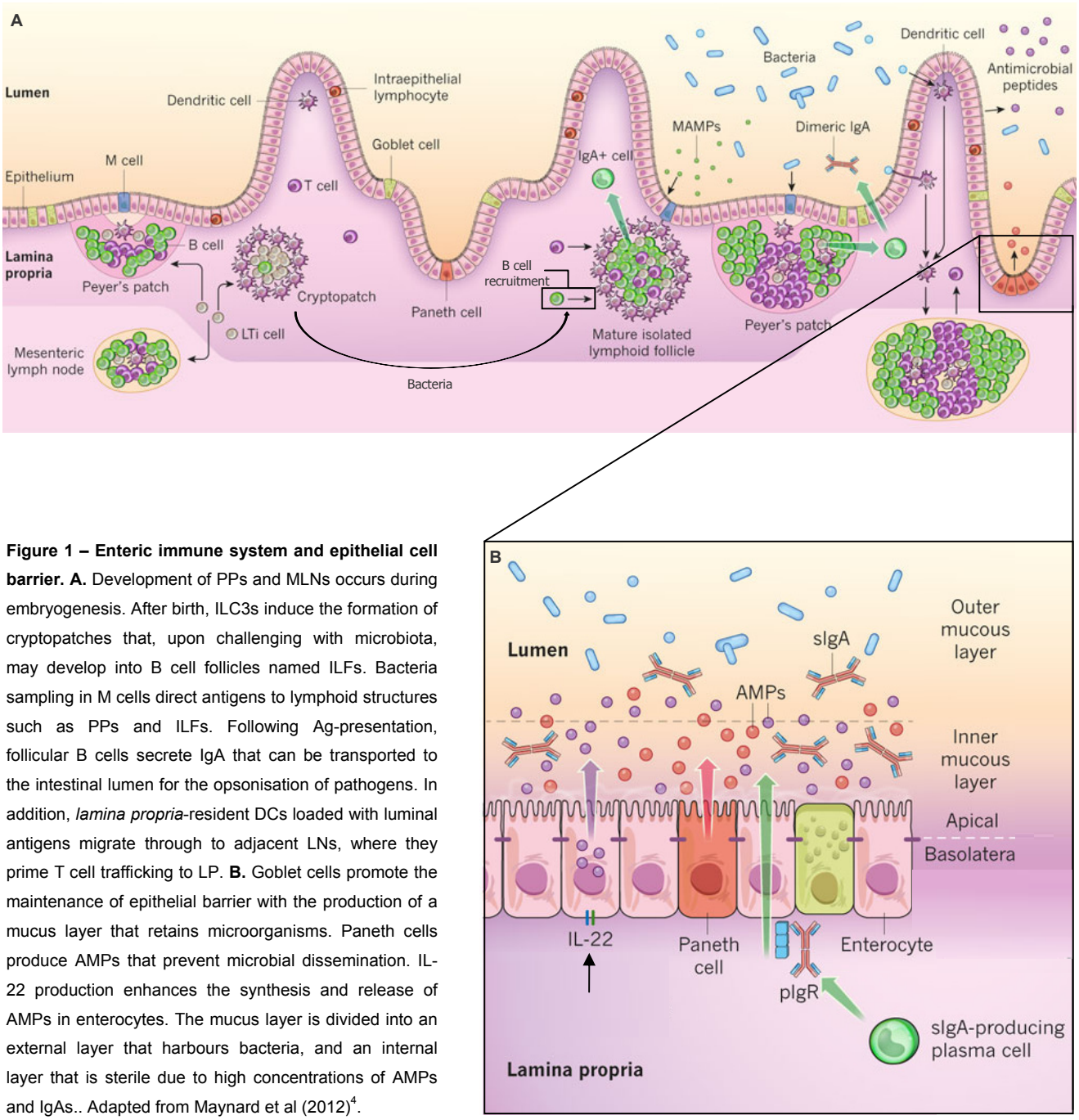

Figure 1 – Enteric immune system and epithelial cell barrier. A. Development of PPs and MLNs occurs during embryogenesis. After birth, ILC3s induce the formation of cryptopatches that, upon challenging with microbiota, may develop into B cell follicles named ILFs. Bacteria sampling in M cells direct antigens to lymphoid structures such as PPs and ILFs. Following Ag-presentation, follicular B cells secrete IgA that can be transported to the intestinal lumen for the opsonisation of pathogens. In addition, *lamina propria*-resident DCs loaded with luminal antigens migrate through to adjacent LNs, where they prime T cell trafficking to LP. **B.** Goblet cells promote the maintenance of epithelial barrier with the production of a mucus layer that retains microorganisms. Paneth cells produce AMPs that prevent microbial dissemination. IL-22 production enhances the synthesis and release of AMPs in enterocytes. The mucus layer is divided into an external layer that harbours bacteria, and an internal layer that is sterile due to high concentrations of AMPs and IgAs.. Adapted from Maynard et al (2012)[4].

4. INTRAEPITHELIAL LYMPHOCYTES

The enteric immune system contains diffuse lymphocytes that reside in the *lamina propria* (LPLs) and T cells embedded between basolateral surfaces of ECs called intraepithelial lymphocytes (IELs) [65]. While the LPLs compartment is highly enriched in CD4 T cells, IELs are mainly composed by CD8 T cells [66]. IELs are highly abundant in the epithelium, existing in a proportion of 16 IELs per 100 epithelial cells in the murine jejunum [11]. Their specific location at the epithelium implies that IELs must balance protective responses, in order to preserve mucosal barrier integrity [2]. Indeed, IELs are main effectors in first-line responses preventing bacterial dissemination through the epithelium [2, 11, 67, 68].

Intestinal IELs are almost solely comprised by T cells, with approximately half of the IELs expressing TCRγδ – which contrasts with the low abundance of TCRγδ^{pos} cells normally found in the periphery [2, 69]. The TCRγδ repertoire is characterized by cells that express a single family of Vγ and Vδ chains [70]. The rearrangements in Vγ locus are developmentally programmed, and are then reflected by the time-dependent emergence of γδ cells that display specific Vγ chains in particular peripheral tissues [71, 72]. In concern of IEL compartment, γδ cells are majorly composed of Vγ7-expressing cells [69, 73]. IELs can also express TCRαβ and can either be CD4^{pos}, CD8^{pos} or negative for both [2, 65].

IEL development has been subject of debate over the years [2]. CD8αβ^{pos} and CD4^{pos} TCRαβ IELs which are denominated induced IELs due to their post-thymic activation nature with peripheral Ags, derive from T cells that underwent conventional thymic selection[2]. γδ cells and CD8αα^{pos} TCR αβ^{pos} are named natural IELs due to their thymus-acquired activated status derived from self-antigen exposure. However, the development of CD8αα^{pos} IELs is still unknown matter of debate (Figure 2) [2].

4.1. T cell development

T cell development occurs in the thymus upon arrival of progenitor cells through the blood [74, 75]. Once in the thymus these progenitors are named double-negative cells (DN) due to the absence of the T cell co-receptors CD4 and CD8 [74, 75]. DN cells encompass four different maturation stages defined as DN1, DN2, DN3, and DN4 which are classified in terms of CD44 and CD25 expression [74, 75]. After homing to the thymus DN1 cells (CD44^{pos} CD25^{neg}) expand and retain in the perimedullary cortex (PMC) where transition to DN2 stage (CD44^{pos} CD25^{pos}) occurs [75]. DN2

cells then migrate toward the inner cortex where the first rearrangements in TCRγ and TCRδ happen due to up-regulation of *Rag1* and *Rag2* gene expression [75]. Migration to the outer cortex overlaps with DN3 stage ($CD44^{neg}$ $CD25^{pos}$) differentiation, where irreversible T cell commitment to a $TCR\gamma\delta^{pos}$ cell (γδ T cell) or a $TCR\alpha\beta^{pos}$ cell (αβ T cell) takes place [74, 75].

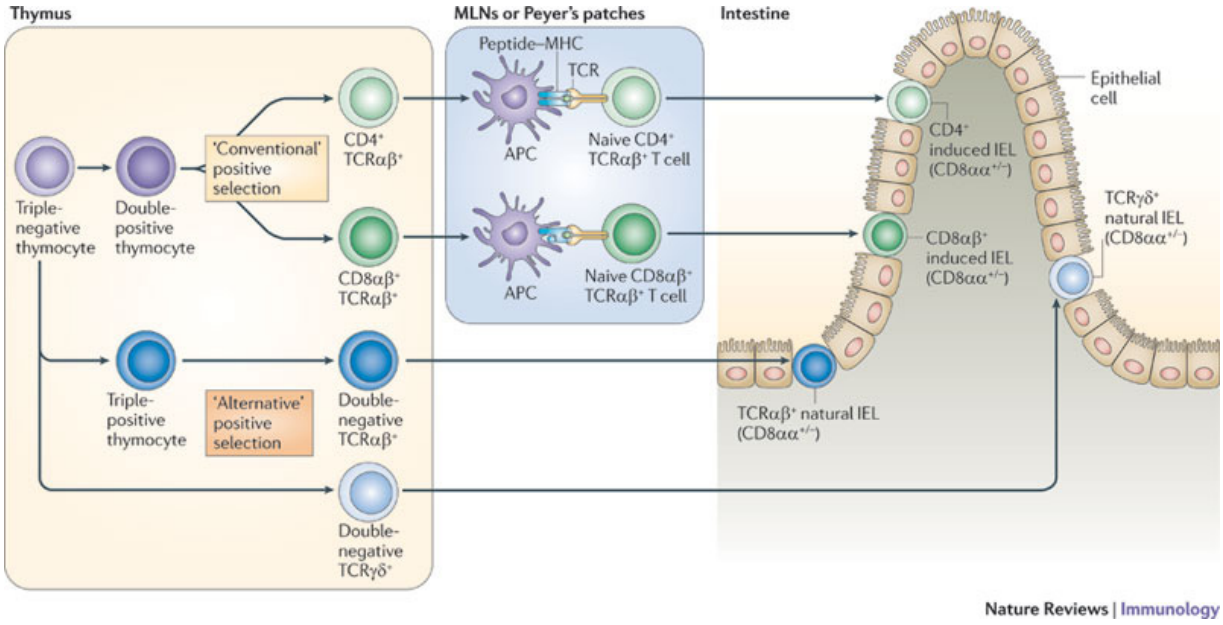

Figure 2 – Thymic selection of IEL subsets. Unconventional IELs derive from double negative thymocytes and migrate to the gut with a pre-acquired activated phenotype. Conventional CD4 and CD8αβ IELs undergo the classical αβ T cell selection at the thymus, and leave to the periphery as naïve T cells. After antigen presentation in the GALT, conventional T cells acquire gut-tropism and migrate to the epithelium, where they are able to reside as IELs.(adapted from Cheroutre 2011 [2])

4.1.1. αβ T cell development

During the DN3 stage, developing thymocytes that express rearranged TCRβ may undergo β-selection[71, 74, 76]. In this transition (DN4 stage : $CD44^{neg}$ $CD25^{neg}$), cells that express a functional pre-TCR – constituted by the pairing of a rearranged TCRβ and an invariant pre-TCRα – proliferate and acquire the expression of CD8 and later the expression of CD4, forming a double-positive (DP) population [71, 76]. Thereafter, rearrangements in TCRα begin, allowing the progression through the double-positive (DP) stage [71]. DP cells undergo positive selection where only adequate TCR-MHCI/II engagement determines T cell survival [74]. After this process,

thymocytes may also undergo negative selection – where cells that avidly engage a self-peptide presented by MHC I or MHC II are eliminated – a crucial checkpoint for the generation of mature $CD4^{pos}$ or $CD8^{pos}$ $TCR\alpha\beta^{pos}$ thymocytes [74]. After development, $TCR\alpha\beta^{pos}$ thymocytes proliferate briefly before leaving the thymus to the periphery [71].

4.1.2. γδ T cell development

Although the development of αβ T cells is well described, much less is still known about the mechanisms of selection to a γδ T–lineage[71] – Which is justified in part by the absence of an exclusive marker for γδ T cell progenitors [71]. Alternatively to the conventional model for αβ T cells generation that requires transition through a DP stage, γδ–selection allegedly occurs in DN2 and DN3 stages [74]. Selection to a γδ–lineage was proposed to rely on either TCRγ and TCRδ recombination accomplishment, rather than TCRβ-selection [71]. SOX13 is expressed during all pro-T-cell stages, being only down-regulated after TCRβ acquisition [74, 78, 79] and DN3 cells require the transcription factor SOX13 for γδ T cell differentiation [77].

The molecular mechanisms ruling the T lineage choice during T cell development have been subject of debate. During the course of time, it became more evident that correlation between the acquisition of a TCR type and the T-lineage fate was not always applicable[80]. For example, it has been suggested that TCRαβ is able to mediate both γδ and αβ T-lineage differentiation [71], and that TCRγδ expression is compatible with both T cell fates [71]. Furthermore, previous studies using TCR transgenic mice reported that an impairment in TCR signalling correlated with decreased DP cells [71] and a decrease in TCRγδ signalling strength in transgenic mice led to an increase of DP cells and a decrease of TCRγδ cells with mature phenotype [81]. These results led to the development of signal strength hypothesis as a lineage determining factor [71]. This hypothesis proposes that weak TCR signalling promotes differentiation to a αβ T cell, while strong TCR signals induces a γδ T cell-like phenotype [71]. In γδ–selection, signal strength would be a crucial checkpoint for the deletion of cells with suboptimal TCRγδ signalling [71].

4.2. IELs and migration

On the one hand, migration of conventional IELs is believed to occur throughout life and to be spurred upon acquisition of gut-homing markers CCR9 and $\alpha_4\beta_7$ [2]. CCR9 interacts with CCL25, constitutively expressed in intestinal ECs [69, 82] and $\alpha_4\beta_7$ integrin allows interaction with mucosal vascular addressin cell adhesion molecule 1 (MADCAM1) present in intestinal stromal cells [69,

[83]. Naive T cells that migrate from the thymus to the periphery may encounter gut-derived DCs that can imprint them with homing receptors (Figure 2) [83, 84]. α_E integrin, also known as CD103, assembles with β_7 integrin to interact with E-cadherin on the basolateral surface of ECs, enabling IEL maintenance in the epithelium [2, 68, 77, 85]. Therefore, α_E constitutes the main adhesion molecule involved in IEL compartmentalization, and enhancement of its expression down-regulates α_4 in cells homing to the intestinal epithelium [2, 68, 77, 85].

On the other hand, migration of unconventional IELs occurs during the first three weeks of life, and it is believed that their gut-tropism imprinting takes place at the thymus, as early $CD8^{pos}$ thymocytes (early thymic migrants – ETM) express $\alpha_E\beta_7$ and CCR9, and are responsive to CCL25 chemotaxis [2, 69, 82, 86, 87, 88]. In fact, increasing evidence supports the notion that acquisition of gut-homing markers may also occur in the thymus, since previous reports identified CCR9, integrin $\alpha_4\beta_7$ and integrin α_E expression in recent thymic emigrants (RTEs – T cells that leave the thymus to circulation) [69]. Recently, Cheroutre *et al*. described $V\gamma7^{pos}$ IELs as being programmed to 'directly' home the gut, thus not requiring the passage through the GALT in order to acquire gut-tropism [2]. Intrathymic induction of tissue-homing markers in T cells that express Vγ7 chains may account for the increased proportion of $V\gamma7^{por}TCR\gamma\delta$ in the gut [89].

Lastly, Guy-Grand *et al* recently published an article identifying the circulating pool of gut-tropic T cells to the intestinal epithelium [69]. They proposed that all T cells leave the thymus with acquired ability to migrate directly to the periphery and the gut, suggesting that mechanisms ruling the tropism of unconventional and conventional IELs may be similar [69]. All these studies reflect how the migratory dynamics of IELs are still much undefined.

4.3. IELs and enteric immune responses

γδ T cells are key components of host protection in the intestine. Intestinal γδ T cells function has been linked with the regulation of enterocyte turnover and antibody class switching and IgA production [2]. In resemblance to other peripheral γδ T cells, TCR repertoire of γδ IELs is restricted [2, 90]. Still, they may be able to respond promptly to invariable bacterial antigens [2, 72]. Upon stimuli, they are able to produce the inflammatory cytokines TNF-α and IFNγ [2, 65, 69, 90], and to secrete high levels of granzyme B and perforin, exhibiting a cytotoxic phenotype [69]. In the other hand, γδ T IELs produce numberless cytokines important for the regulation of immune responses and epithelial barrier protection [2, 65, 77, 91]. Among those are TGFβ1, TGFβ3, and

keratinocyte growth factor (KGF) [77, 92]. Recently it was also shown that γδ T cells produce IL-22, hence suggesting that they may lead to attenuation of intestinal inflammation [62].

CD8αα is considered an activation marker for T cells since its expression levels rely on CD3 activation [2]. CD8αα is unable to support positive selection of MHC-I restricted thymocytes [93] and activation of MHC-I-restricted IELs [2]. Consequently, CD8αα suppresses TCR signalling and may set a higher threshold for TCR activation [2]. Nevertheless, CD8αα^{pos} αβ T lymphocytes display cytotoxic potential with the expression of granzyme B and FASL [2, 91]. Additionally, their responsiveness to self-antigens indicates a potential antitumor role [2].

Conventional CD8αβ^{pos} αβ T IELs are antigen-experienced T cells [2]. They display characteristics common to peripheral memory T cells such as long-lasting immune responses upon repeated challenging [2, 68].

Lastly, although most epithelial T cells are CD8^{pos} and *lamina propria* T cells are CD4^{pos}, some IELs are CD4^{pos} as well [2]. Alike other IELs, CD4 αβ T IELs play important roles in protective immunity [2], through the production of TNF-α and IFNγ [94]. However, since the intestinal milieu favours the establishment of regulatory immune responses, CD4 αβ T IELs may also contribute for the control of inflammatory responses [2].

5. INNATE LYMPHOID CELLS

5.1 Subsets and effector functions

Innate lymphoid cells (ILCs) are a heterogeneous family of haematopoietic cells that mediate the development of innate immune responses upon pathogen challenging during adulthood [3, 18, 34, 52, 95, 96, 97, 98, 99, 100]. They also appear to contribute to mucosal tissue integrity, remodelling and to the anatomical containment of commensal bacteria [42, 52, 97, 98, 100, 101, 102].

ILCs have a lymphoid morphology, lack rearranged antigen receptors and their development is independent of *Rag* genes [103]. are grouped in three main categories: ***i.*** Group 1 ILCs: include ILC1 and natural killer cells (NK cells) which depend on T-bet, IL-7 and IL-15 and produce IFNγ [3]; ***ii.*** Group 2 ILCs: are RORα and GATA3 dependent, IL-7 dependent that rapidly respond against parasitical infections through the production of IL-5 and IL-13 [3, 104]; ***iii.*** Group 3 ILCs are RORγt dependent, rely on IL-7 and produce IL-17 and IL-22 (Figure 3) [3, 104]. More recently, it was also shown that AhR drives RORγt ILC3s and CP development [105, 106, 107].

LTi cells represent the prototypical subset of group 3 ILCs and play a crucial role during development of secondary lymphoid organs in the embryo [24]. In the embryo, they are known for their CD45^{pos} RORγt^{pos} CD4^{pos} LTα^{pos} LTβ^{pos} CD127^{pos} CD117^{pos} phenotype [108]. In adulthood they mature into LTi-like cells and are capable of producing IL-22 and IL-17 [104]. As aforementioned, they are indispensable for the development of CPs, and consequently ILFs [21]. Additionally, it was reported that LTi-like cells may be fundamental in the reconstruction of LN after acute viral infection. However, their exact immune function in adulthood is still unknown [3, 99, 100].

Analysis of RORγt^{pos} ILC3s revealed diverse functional subsets in addition to LTi cells. Some ILC3 express the natural cytotoxicity triggering receptor (NCR) NKp46 and were named NCR^{pos} ILC3s [3]. Unlike NK cells, NCR^{pos} ILC3s do not display cytotoxicity or produce IFNγ [109]. NCR^{pos} ILC3 development also depends on the transcription factor T-bet [110]. NCR^{pos} ILC3s express IL-22 but not IL-17 [100]. IL-22-producing ILC3s are critical for the innate immune response against attaching-effacing bacteria, such as *Citrobacter rodentium* [109, 111, 112, 113]. IL-22 produced by this subset acts on gut epithelial cells inducing the production of anti-microbial peptides, thus these cells have been implicated in early immune responses to attaching and effacing intestinal infections [63, 114]. Another ILC3 subset lacks NCR expression and produces IFNγ and IL-17 in

addition to IL22 and were named NCR^{neg} ILC3s[3] (Figure 3). NCR^{neg} ILC3s are believed to mediate pathology in innate colitis mouse models and to be increased in IBD patients [42, 115, 116].

ILC3s may also critically regulate CD4-mediated immune responses [102]. A MHC-II expressing population of ILCs was recently described and shown to limit commensal-driven immune responses, independently of IL-17 or IL-22 production [102]. Furthermore, it was recently shown that ILC3s were pivotal for the containment of *Candida albicans* infection in mice [117]. IL-17 production by ILCs, was able to control the fungal infection even in mice deficient in B and T cells [117]. Additionally, depletion of ILCs resulted in failure of fungal suppression, revealing a crucial role of ILC3s in cytokine-mediated immune responses at the onset of infections [117].

5.2 Group 3 ILC development

During ILC development, Id2 expression is acquired in the CLPs, allowing the selection to a ILC-lineage specification [17, 118, 119]. Herein, Id2 inhibits the E2A transcription factor, disabling the B cell potential of CLP [17]. After that, Id2^{pos} ILC precursors become able to generate all group 1 and group 2 ILC [100]. The developmental plasticity between distinct ILCs population are still largely unclear [100]. In this regard, two main hypotheses concerning the developmental nature of ILC3 cells were formulated. One defends that ILC3s are the progeny of foetal LTis [100]. The alternative hypothesis defends that ILC3s derive from CLP-like cells that do not express $\alpha_4\beta_7$, rather than from Id2^{pos} ILC precursors that express $\alpha_4\beta_7$ [100, 120]. Still, much more remains to be discovered about ILCs development in order to assess its developmental pathways [3, 17, 100, 119].

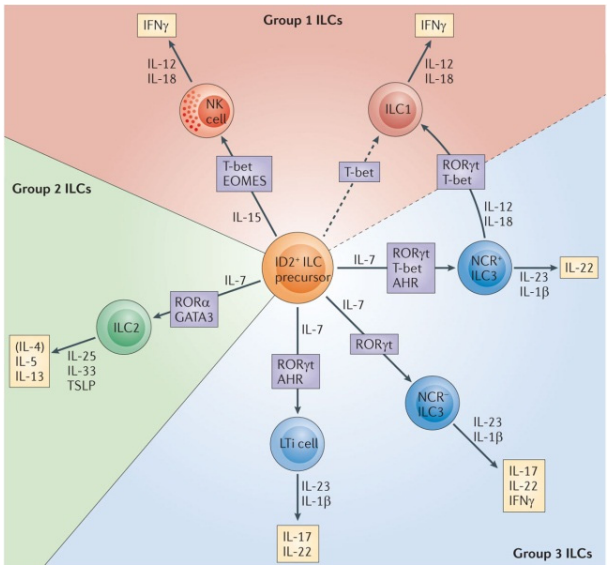

Figure 3 – Transcriptional programs and cytokine profiles of ILCs families . Group 1 ILCs, NK cells and ILC1s require T-bet for their development and function and express IFNγ. Group 3 ILCs, LTi cells (LTi4 in the adult), NCR^{pos} ILC3 and NCR^{-} ILC3s (LTi0) development highly depends on RORγt expression, as well as their function. They all express IL-22, but LTi4 cells and LTi0 express IL-17 and LTi0 also expresses IFNγ. Lastly, Group 2 ILCs, require GATA3 for their development and express typical Th2 cytokines.(adapted from Spits 2013 [3])

6. RETINOIC ACID AND IMMUNITY

Vitamin A (or retinol), is a lipophilic factor required for the formation of most tissues during embryogenesis[121, 122]. However, mammals are not able to synthesize retinol and therefore all retinoids are obtained through the diet. Retinol is found in animals in the form of retinyl esters and in plants in the form of carotenoids. After intake, retinyl esters are metabolized into retinol in the intestinal lumen by retinyl ester hydrolases (REHs) [123]. Retinol is then oxidised into retinaldehyde by retinol dehydrogenases (ADHs) and converted in retinoic acid (RA) by retinaldehyde dehydrogenases (ALDHs or RALDHs) [1, 122, 123, 124].

RA is the biologically active metabolite of retinol [1, 122, 123, 124]. It interacts and activates signalling events through the binding of retinoic acid receptors (RAR), a family of transcription factors that regulate gene expression [122, 123, 125, 126, 127, 128]. The transcriptional regulation mechanism exerted by RARs relies on dimerization with retinoid-x-receptors RXR and subsequent DNA binding to specific sequence elements named RA response elements (or RAREs), located in the promoters of target genes (Figure 4). The RAR family is encoded by 3 genes, *Rara, Rarb,* and *Rarg* [128]. RARα, encoded by *Rara* is amidst RAR subtypes the most ubiquitously expressed [129]. *Rara* knockout mice exhibit various malformations, which suggest a crucial role of this receptor in the transduction of retinoid signalling during early embryonic development [122, 123, 125, 126, 127, 128].

6.1 Role in lymphocyte migration and immune responses

Vitamin A deficiency affects approximately 250 million people worldwide, increasing childhood susceptibility to pulmonary and gastrointestinal infections, probably due to deficient protection of the epithelial barrier[129]. Adequate mucosal immune responses require rapid migration of activated T cells to the site of infection, and RA induces the expression of gut homing markers, enabling them to migrate and reside within GALT-tissues. Specifically, gut-derived DCs produce RA from vitamin A uptake, and provide it to T cells inducing expression of the gut-homing markers CCR9 and $\alpha_4\beta_7$ (Figure 4) [121]. Iwata *et al.* demonstrated that vitamin A-deficient diet depletes both $CD4^{pos}$ and $CD8^{pos}$ T cells from murine intestines[84]. This further evinced the importance of retinoic acid in T lymphocyte tropism to the gut, but it remains to be determined if RA is a cell-autonomous requirement to these cells.

Retinoic acid has been extensively related with the regulation of immune responses in T cells, since it enhances the expression of Foxp3, transcriptional factor responsible for the

transcriptional program of regulatory T cells – Tregs (Figure 4) [124, 130, 131, 132, 133, 134]. On the other hand, establishment of protective CD4 T cell responses is highly dependent on RA signalling events [129]. Vitamin A deficiency results in impaired mucosal Th1 and Th2 immunity, which can only be reversed by RA supplementation [126, 127]. Interestingly, RA also promotes the production of IL-17 in CD4 and CD8 splenic T cells *in vitro* (Figure 4) [135]. Recently, Mills *et al.* demonstrated that RA administration was able to induce IL-22 production in γδ T cells and ILCs {Mielke, 2013 #13}. Thus, RA is exceedingly important for the regulation of T cell-mediated responses.

Diet-derived nutrients, such as vitamin A, have been extensively related with the immunomodulation of T cell responses and lymphocyte migration. Therefore, it is crucial to disclose the mechanisms by which RA orchestrates the development of the enteric immune system.

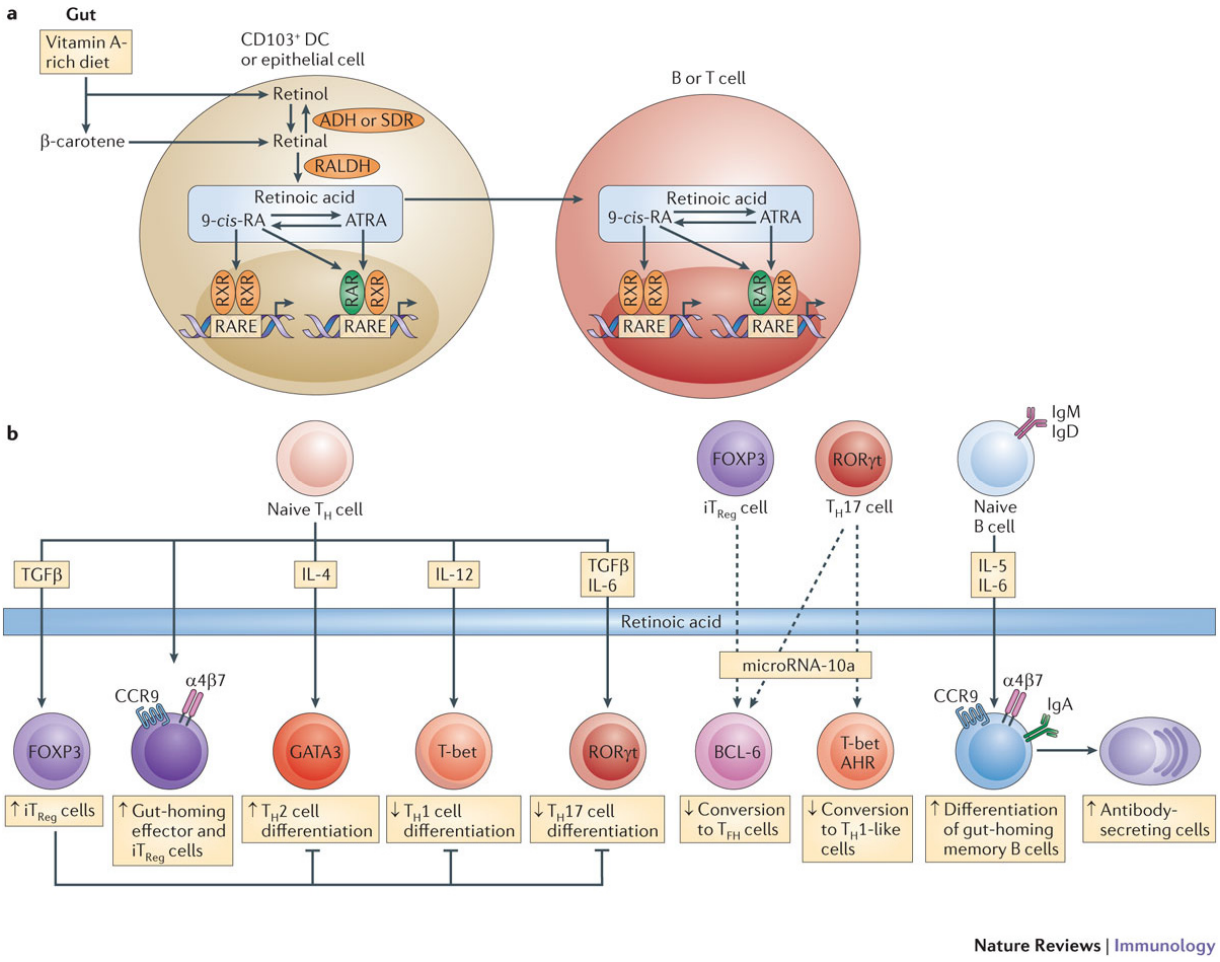

Figure 4 – Functions elicited by RA in the gut immunity. CD103^{pos} DCs metabolize dietary Vitamin A to produce retinoic acid. Then, RA is provided to T or B cells and elicits diverse functions crucial for enteric immunity and lymphocyte migration. (adapted from Veldhoen 2012 [1])

7. AIMS OF THIS THESIS

The intestinal mucosa is endowed with a specialized immune system, comprised by adaptive T and B lymphocytes and innate lymphocytes. Diet-derived retinoic acid has emerged in the last years as an important factor for the regulation of T and B cell establishment and responses in the intestinal *lamina propria*. Nevertheless, its relevance for the immunomodulation of lymphocytes that reside in the intestinal epithelium (IELs) has never been described. In addition, the factors regulating innate lymphoid cells (ILCs) development and maintenance in the intestine are still very elusive. In this thesis, we intended to study the role of retinoic acid signalling in the regulation of the enteric immune system.

With the purpose of determining the impact of retinoic acid (RA) signalling in IELs and ILCs, we used a mouse model with deficient RA signalling in all T cells and ILCs. With this strategy, we assessed the importance of RA signalling in IEL homeostasis and function by flow cytometry and gene expression analysis. We then identified the stages of αβ T cell development in which RA impact occurs. To do so, we used a mouse model with deficient RA signals in all αβ T cells and after the development of ILCs. We also assessed if RA impacts ILCs function, maintenance and expansion after their development, using flow cytometry, molecular biology, proliferation assays and an inflammation model; with the same mouse model. With the aim of determining if RA could be involved in the development of lymphoid structures after ILCs development we observed lymphoid structures at the LP using confocal microscopy.

With our work we aim for a better comprehension of the mechanisms ruling the establishment and maintenance of the enteric immune system and the immunomodulation of T cells at the intestinal epithelium.

V. MATERIAL AND METHODS

1. MOUSE STRAINS

CD2-cre[136] and *Rorc-cre*[21] mice heterozygous and homozygous for *RaraT403* mutation[137] and *CD2cre R26*-YFP[136] and *Rorc-cre R26-GFP* mice[138] were on a C57BL/6J genetic background. All mouse strains were bred and maintained at the IMM animal facility. All procedures and experiments were performed accordingly to institutional and national guidelines.

2. GENOTYPING PCR

DNA isolation was performed by briefly keeping of snipped tails in Tail Lysis Buffer (Annex 2) and 0.4mg/mL of Proteinase K at 56ºC until the tissue was completely digested. Then, DNA was extracted by centrifuging the samples with Isopropanol for 25 min 4º C and 13000rpm, and by washing the pellet with 70% Ethanol followed by centrifugation at 13000rpm for 10 min at 4º C. DNA was ressuspended in Milli-Q water.

DNA amplification was performed on a Veriti 96-Well Thermal Cycler (Applied Biosystems). The PCR program used consisted of an initial step of polymerase heat activation, performed at 95 ºC during 10 min, followed by 35 amplification cycles consisting of 3 distinct steps (DNA denaturation – 94º C for 30 sec, DNA-primer annealing step – 60º C for 45 sec, and polymerase reaction elongation – 72ºC for 1 min.), and a final step of DNA extension at 72º C for 10 min. Primers sequences are detailed in Annex 3. To each 19µL of PCR buffer (Annex 2) 1µL of DNA was added to individual wells. After PCR reaction amplified products were resolved in a 1.5% agarose gel containing Gel Red (Biotium).

3. MOUSE ANALYSIS

For adult IELs, LPLs and ILCs isolation, 8-weeks old mice were dissected and the intestines removed. After flushing the intestines content with PBS (Annex 2), Peyer's patches were removed and the intestines cut longitudinally from the mesenteric side and in smaller pieces of 1-2 cm. IELs were obtained by stirring the guts with IELs isolation medium (Annex 2) for 2

rounds of 20 minutes and extracted cells were filtered. Hereafter, *lamina propria* lymphocytes and ILCs were obtained by digesting the guts with digestion medium (Annex 2) for one round of 15 min and another of 10 min. After each digestion, isolated cells were filtered into individual tubes. IELs and *lamina propria* lymphocytes were then purified by using a 40-80% percoll gradient with complete medium and centrifuging for 30 min, 2400 rpm at room temperature. Cell pellets were then ressuspended with 300 µL of complete medium (Annex 2). For the isolation of IELs from young mice, neonates and 15-day olds were sacrificed and dissected. Whole guts were flushed and digested with digestion medium as aforementioned. Purification of lymphocytes was performed as in adult mice. Thymi and spleens were extracted and lymphocytes obtained by tissue homogenization through cell-meshes.

4. STAINING AND FLOW CYTOMETRY

Isolated cells were stained in 96-well plates. For staining of surface markers, cells were initially incubated with Fc block in FACS buffer (Annex 2), in order to prevent binding of used antibodies (Ab) by Fc receptors. Cells were stained with respective Ab mixes (Annex 4). All staining steps were performed in ice and in the dark. For staining of nuclear factors, cells were fixed in Fixation Buffer (eBiosciences) for 20 min and permeabilized in 1X Perm Buffer (Annex 2) for 5 min at room temperature. Cells were then incubated with RORgt (AFKJS-9) for 30 minutes and washed in Perm Buffer. For cytokine staining, cells were previously incubated with an activation medium (Annex 2) for 4h 30min at 37º C, in order to activate cytokine production in cells and block their secretion. Data analysis was performed using FlowJo software. Used Abs were: anti-Vγ7 (F2.67 Pereira), anti-δ TCR (GL3), anti-NKp46 (29A1.4), anti-RORγt (AFKJS-9), anti-IL-17 (eBio17B7), anti-IFNγ (XMG1.2), anti-CD4 (RM4.5), anti-TCRβ (H57-597), anti-CCR9 (CW-1.2), anti-Thy1.2 (53-2.1), anti-CD45 (30-F11), anti-B220 (RA3-6B2), anti-CD19 (MB19-1), anti-CD3 (eBio500A2), anti-Gr-1 (RB6-8C5), anti-CD11c (N418), anti-TER119 (TER-119), from eBiosciences; and anti-CD8b (4TS156.7.7), anti-CD8a (53-6.7) from Biolegend.

5. GENE EXPRESSION ANALYSIS

RNA was isolated using RNAeasy Micro Kit (Qiagen), accordingly to manufacturer's protocol and extracted RNA samples were stored at -80º C. RNAs were converted in cDNA with reverse transcriptase PCR, cDNAs pre-amplified and amplified with specific probes (qPCR) using a

Veriti 96-Well Thermal Cycler (Applied Biosystems). RT-PCR, pre-Amp PCR and qPCR assays were performed accordingly to manufacturer's protocol (Applied Biosystems). Used probes are detailed in Annex 3. All samples were run in a StepOne Thermal Cycler (Applied Biosystems) and results were analysed with StepOne software (Applied Biosystems).

6. GUT WHOLE-MOUNT IMAGING

8-weeks old mice intestine were washed with PBS 1X, and opened through the mesenteric side. A portion of 3 cm was cut from the end of the jejunum. Mucus and epithelial cells were scraped off with coverslips, and washed again in PBS 1X. After washing, samples were fixed with 4% PFA in PBS (Annex 2) for 10 min at 4º C. Intestines were then incubated with Block Solution (Annex 2) during 1h at 4º C. Then, guts were incubated with primary Abs overnight at 4º C. Intestines were incubated with a secondary Ab coupled with a fluorophore for 6h at 4º C and washed with Blocking Solution. After staining, intestines were gradually dehydrated in successive concentrations of methanol in Block Solution of 25%, 50%, 75% and 100% for 5 min and another cycle of 100% for 1h, at room temperature. Thereafter, guts were cleared with BABB solution (Annex 2) for 10-15 min at room temperature. Finally, guts were placed in depression slides with BABB, and sealed with coverslips, and analysed using a Zeiss LSM 710 confocal microscope.
Used Abs were: anti-RORγt (AFKJS-9), Alexa Fluor 647 anti-B220 (RA3-6B2) from eBiosciences and Alexa Fluor 488 anti-IgG from Invitrogen.

7. CELL-CYCLE AND CELL-DEATH ANALYSIS

8 weeks old mice were injected intraperitoneally twice with 66.6 µg of BrdU per 1g of its weight, 36h and 12h before cell-cycle analysis. After IEL and ILC extraction, surface and intracellular staining with anti-BrdU (3D4) were performed with BrdU Flow Kit (BD Pharmingen) as described in the manufacturer's protocol. For cell-death analysis, extracted ILCs from 8 week old mice were stained with FITC-coupled Annexin V in Annexin Buffer (eBiosciences). All cell preparations were analysed using a BD Fortessa flow cytometer (BD biosciences).

8. STATISTICS

Statistical analysis was performed using two-tailed *F*-test analysis of variance and two tailed Student's *t*-test. A *p*-value of <0.05 was considered significant. Results were scored as * when $p<0.05$, ** when $p<0.01$, and *** when $p<0.001$.

VI. RESULTS

1. RA SIGNALLING DEFINES INTESTINAL T CELL NUMBERS

Retinoic acid is a diet-derived metabolite that plays important roles in the immune system [84, 121, 122, 123, 124]. Previous studies reported that vitamin A deficiency results in reduced CD4 and CD8 T cell numbers in the intestinal LP [84], suggesting that RA signalling might be also a critical requirement for enteric IELs. In order to test this hypothesis, we used a mouse line with a truncated form of RARα knocked into the *ROSA-26* locus (*T403* mice)[137]. This RARα mutant terminates after amino acid 403, and acts as a dominant negative: it is irresponsive to RA and inhibits endogenous RAR forms[137]. *T403* mice were bred to mice expressing *Cre* recombinase under control of the human *Cd2* regulatory elements[136] (*CD2cre* mice). In these mice, *Cre* expression is observed in most early DN1 thymocyte progenitors[136], and should be present in virtually all T cells. Accordingly, analysis of *CD2cre-R26-YFP* reporter mice revealed that all conventional and unconventional IELs are YFP^{pos}, demonstrating that these lymphocyte subsets have undergone *Cre* recombination (Annex 1 – Figure S1A).

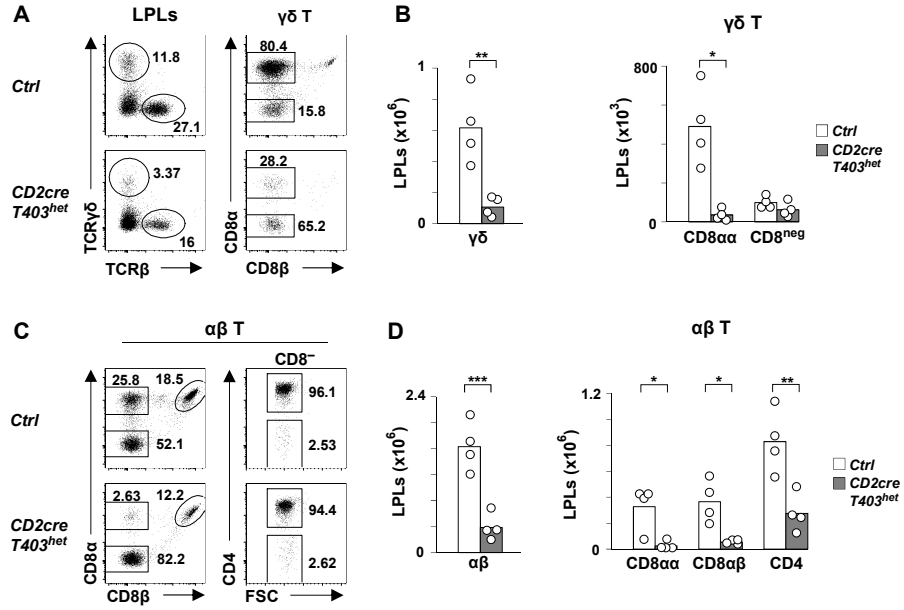

Figure 5 –Lymphocyte autonomous RA signalling controls LPLs. LPLs were obtained from the guts of 8 weeks old *CD2cre T403^{het}* and *wt* litter-mate control mice. **A.** Representative FACS analysis plots for total LPLs and γδ LPL subsets. **B.** γδ LPL subsets cell numbers. **C.** Representative FACS plots for αβ LPL subsets. **D.** αβ LPL subsets cell numbers. *Ctrl* n=4; *CD2cre T403^{het}* n=4. *p<0.05, **p<0.01, and ***p<0.001.

We found that lymphocyte-autonomous RA signalling blockage led to reduced γδ and αβ LPL subsets (Figures 5A, B, C and D), in agreement with previous reports using dietary RA. Most importantly, analysis at 8 weeks of age revealed that enteric IELs were profoundly reduced in

CD2cre T403 animals (Figure 6). This decrease was transversal to conventional and unconventional TCRγδ^{pos} T cell subsets, including both foetal-origin Vγ7^{pos} IELs and their adult Vγ7^{neg} counterparts (Figures 6A and C). Similarly, *CD2cre T403* mice had reduced numbers of conventional and unconventional CD8 TCRαβ IELs, but normal CD4 TCRαβ IELs (Figure 7). Our data demonstrate that RA is a major regulator of IELs in a lymphocyte-autonomous manner.

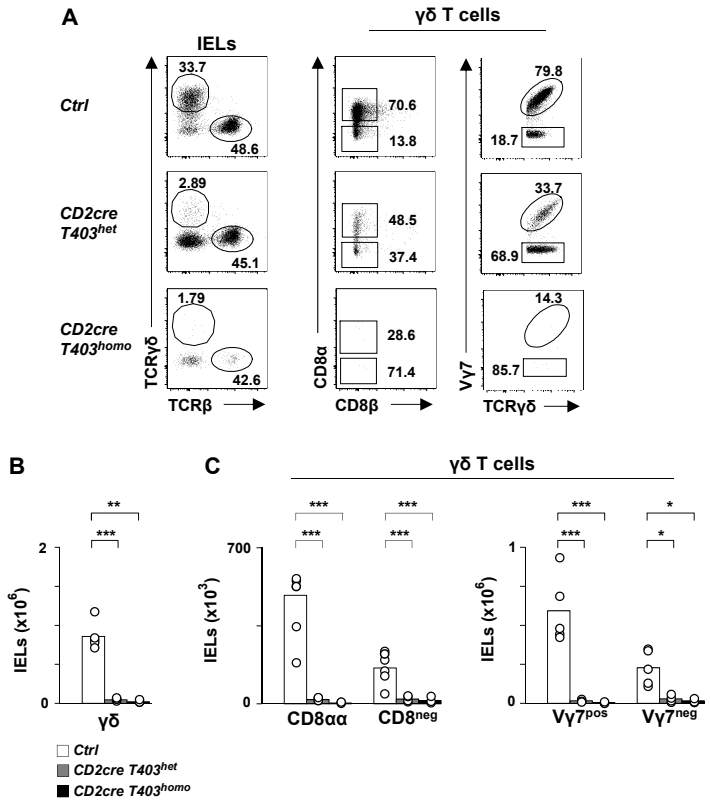

Figure 6 – Lymphocyte autonomous RA signalling controls enteric γδ T IELs. Intraepithelial lymphocytes were obtained from the guts of 8 weeks old *CD2creT403^{het}* and their WT litter-mate controls. **A.** Representative flow cytometry (FACS) plots for total IELs and for γδ IEL subsets (identified by staining of CD45 and TCRδ). **B.** γδ IEL cell numbers. **C.** CD8αα and CD8^{neg} γδ IEL subsets cell numbers and Vγ7-expressing γδ and Vγ7^{neg} IEL cell numbers. *Ctrl* n=4; *CD2cre T403^{het}* n=6; *CD2cre T403^{homo}* n=3. *p<0.05, **p<0.01, and ***p<0.001.

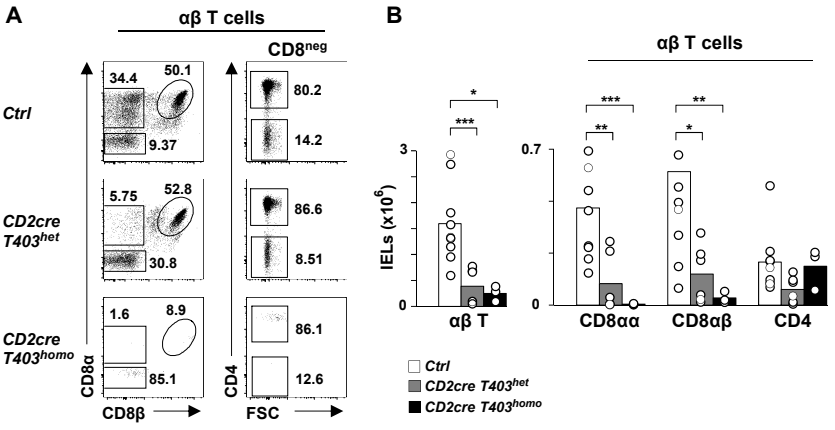

Figure 7 – Lymphocyte autonomous RA signalling controls enteric αβ T IELs. A. Representative FACS plots for αβ IEL subsets (identified by staining of CD45 and TCRβ). **B.** αβ IELs cell numbers. *Ctrl* n=7; *CD2cre T403^{het}* n=6; *CD2cre T403^{homo}* n=3. *p<0.05, **p<0.01, and ***p<0.001.

The marked reduction of enteric *CD2cre T403* T cells led us to evaluate if this deficiency was secondary to a T cell production deficit. Previous studies reported that $CD8^{pos}$ thymus migrants start colonizing the gut in the first three weeks after birth[86], thus we evaluated T cell numbers in the thymus and gut in two weeks old *CD2cre* $T403^{het}$ mice [88].

The analysis of *CD2cre* $T403^{het}$ mice revealed that, despite normal or even increased numbers of γδ T cell subsets in the thymus, these cells were profoundly reduced in the gut (Figures 8A, B, and C). As an example, *CD2cre* $T403^{het}$ CD8αα γδ and αβ T cells were increased while the enteric IEL counterparts were significantly diminished (Figures 8B and C; and Figures 9A, B and C). Interestingly, *CD2cre* $T403^{het}$ CD8αβ αβ T cells were also increased in the thymus, although they were still founf in seemingly normal numbers in the enteric epithelium (Figures 9A, B and C). Altogether, these results suggest that RA signalling may have a minor impact in T-cell development, while it is critical for a normal pool of most γδ and αβ T IELs.

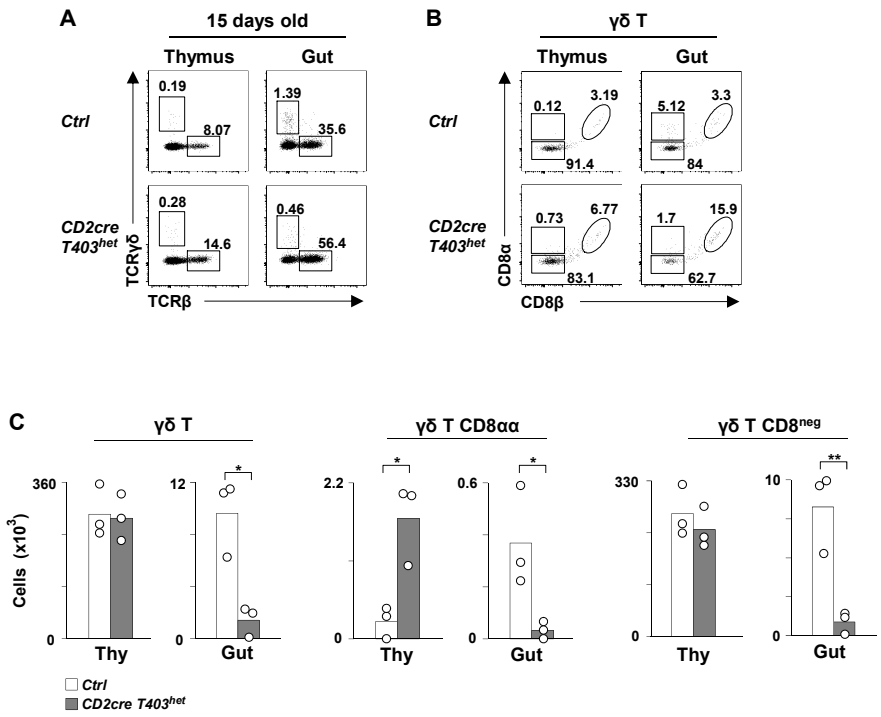

Figure 8 – lymphocyte-autonomous RA signalling and thymic γδ T development. Thymocytes and IELs were obtained from 15-days old *CD2cre* $T403^{het}$ and *wt* litter-mate control mice. **A.** Representative FACS analysis plots for T cells in thymus and gut. **B.** Representative FACS analysis plots for γδ IEL subsets in thymus and gut **C.** γδ T cell numbers in thymus and gut. *Ctrl* n=3; *CD2cre* $T403^{het}$ n=3. *p<0.05, **p<0.01, and ***p<0.001.

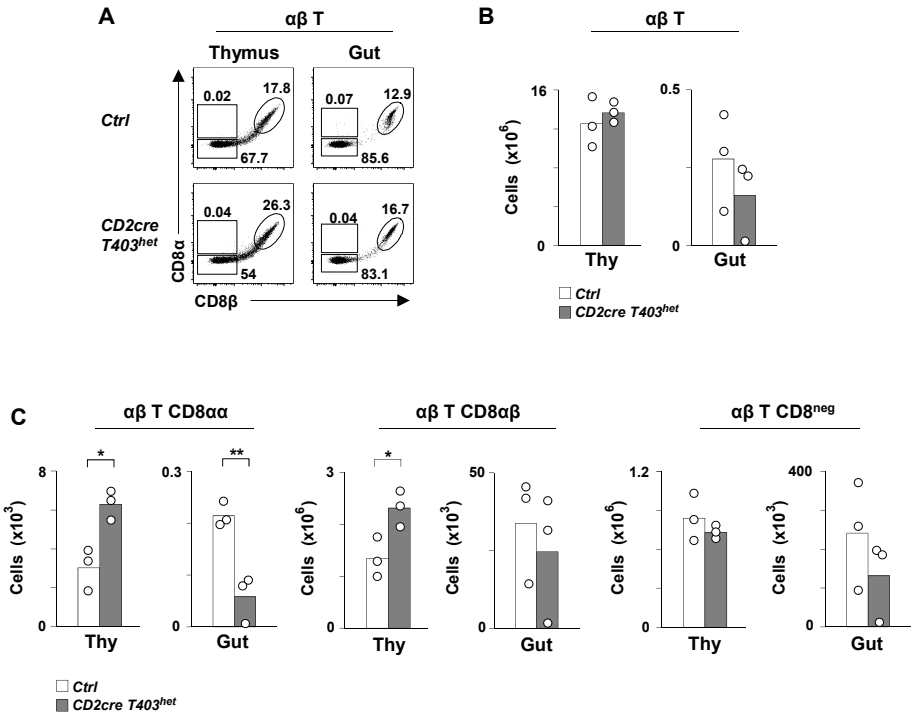

Figure 9 – Deficient cell-autonomous RA signalling leads to CD8αα αβ T and CD8αβ αβ T cell arrest in the 15-day old thymus. Thymocytes were obtained from the thymi and IELs were obtained from the guts of 15-days old *CD2cre T403*^{het} and *wt* litter-mate control mice. **A.** Representative FACS analysis plots for αβ subsets in thymus and gut. **B.** CD8αα αβ T cell numbers in thymus and gut. **C.** CD8αβ αβ T and CD8^{neg} cell numbers in thymus and gut. *Ctrl* n=3; *CD2cre T403*^{het} n=3. *p<0.05, **p<0.01, and ***p<0.001.

2. CELL-AUTONOMOUS RA SIGNALLING IMPACTS CCR9 EXPRESSION IN IELS

In order to determine the cause of IEL reduction in the absence of RA signal events, we questioned if it was either due to an impairment of IEL maintenance, seeding, or proliferation. Firstly, in order to investigate the potential role of RA signalling in IEL steady-state proliferation rate, mice were injected i.p. with BrdU 36h and 12h before analysis. Analysis of *CD2cre T403^{homo}* mice showed that proliferation of both γδ and αβ T IEL subsets was not by reduced RA signalling as revealed by BrdU incorporation (Figure 10).

Sequentially, we analysed IELs at a molecular level. In order to assess whether lymphocyte-autonomous RA signalling could control IEL maintenance, we analysed expression of *Ahr {Li, #1},* and of the anti-apoptotic genes *Bcl2l1* and *Bcl2.* Interestingly, *CD2cre T403^{het}* IELs expressed comparable levels of *Ahr* and similar or even increased levels of *Bcl2l1* and *Bcl2* in most IEL subsets (Figure 11).

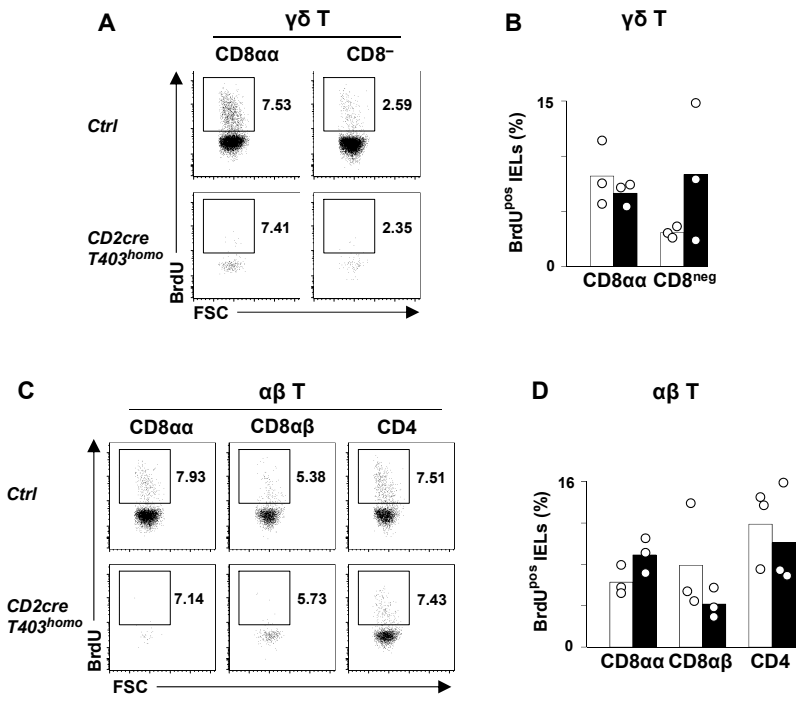

Figure 10– Cell-autonomous RA signalling does not affect IELs proliferation. 8 weeks old *CD2cre T403^{het}* mice and their WT litter-mate controls were injected i.p. with BrdU 36h and 12h before analysis. **A.** Representative FACS plots for γδ IEL BrdU incorporation. **B.** Proportion of BrdU^{pos} γδ T IEL subsets. **C**. Representative FACS plots for αβ T IEL BrdU incorporation. **D**. Percentage of BrdU^{pos} αβ T IEL subsets; Ctrl n=3; *CD2cre T403^{homo}* n=3.

Analysis of the gut-homing markers *Ccr9, Itga4, Itgae* and *Itgb7*, revealed that *CD2cre T403^{het}* IELs displayed reduced *Ccr9* expression (Figure 11). *Itga4* – encoding for α_4 integrin – was also down-regulated in *CD2cre T403^{het}* γδ T cells (Figures 11A and B). Interestingly, *Itgae* and *Itgb7*

encoding respectively for integrins α_E and β_7 – fundamental for IEL retention at the epithelium – were up-regulated in *CD2cre T403*^{het} CD8αα unconventional T IELs (Figures 11A and C).

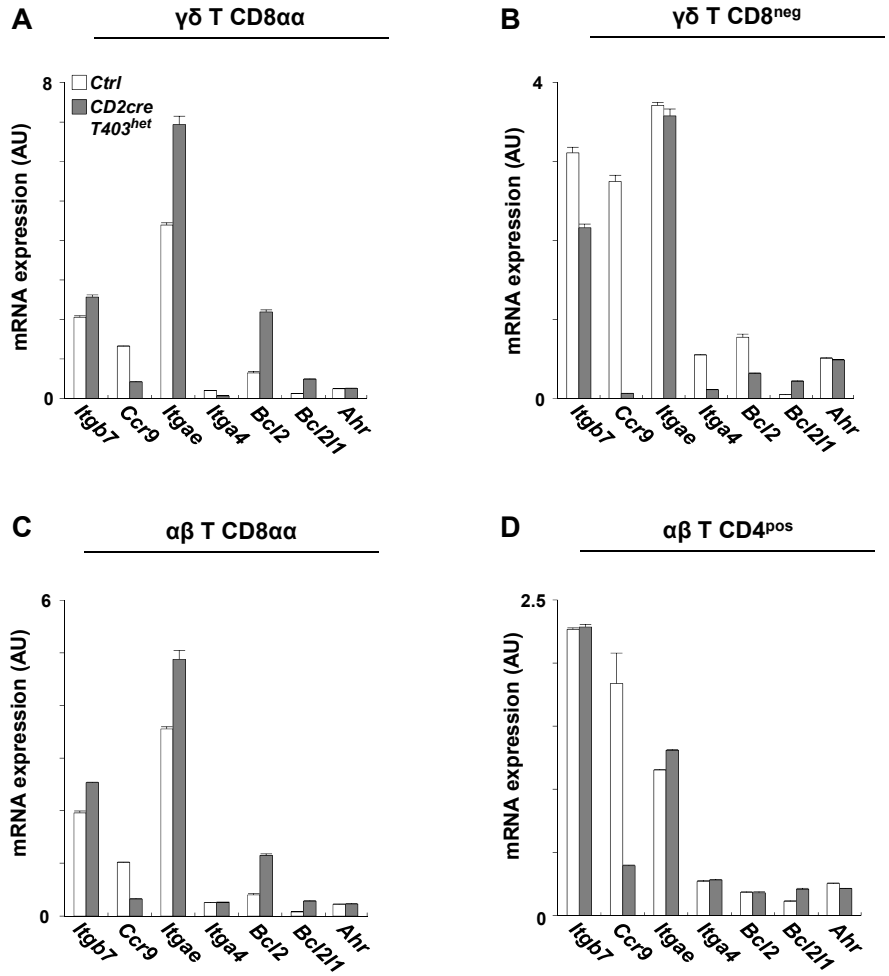

Figure 11 – *CD2creT403*^{het} γδ IELs have decreased CCR9 expression. Enteric IELs were obtained from 8 weeks old *CD2cre T403*^{het} mice and their WT litter-mate controls. Arbitrary mRNA levels on IELs subpopulations. Ctrl n=2; *CD2cre T403*^{het} n=2.

Taken together, our results show that RA signalling is dispensable for IEL proliferation and expression of the IEL survival genes *Ahr*, *Bcl2*, and *Bcl2l1*. Thus our data suggest that reduction of *CD2cre T403*^{het} IELs might be caused by a decreased T cell tropism to the intestinal epithelium rather than by an impairment of IEL maintenance and proliferation. In this concern, RA appears to be pivotal for the acquisition of CCR9 expression, which in turn is fundamental for T cell migration to the intestinal epithelium and may be acquired in the thymus. In addition, *Bcl2l1*, *Bcl2, Itgae* and *Itgb7* up-regulation in *CD2cre T403*^{het} CD8αα^{pos} IELs suggests the existence of a compensatory mechanism to conserve T cell retention in the intestinal epithelium.

3. CONVENTIONAL AND UNCONVENTIONAL αβ T CELLS REQUIRE RA SIGNALS IN DIFFERENT STAGES OF T CELL-DEVELOPMENT

Following the hypothesis that IELs may require RA cues in the thymus for efficient gut-homing, we aimed at defining in which T cell development stage this RA impact occurred. For that purpose, *T403* mice were bred to mice expressing *Cre* recombinase under control of the *Rorc* regulatory elements [21]. Although γδ T cells – which are the progeny of DN thymic precursors and do not pass through the DP developmental stage where *Rorc* is temporarily expressed – should not be affected by the *Rorc-cre* expression [21], all αβ T cells are the progeny of *Rorc* expressing thymocytes.

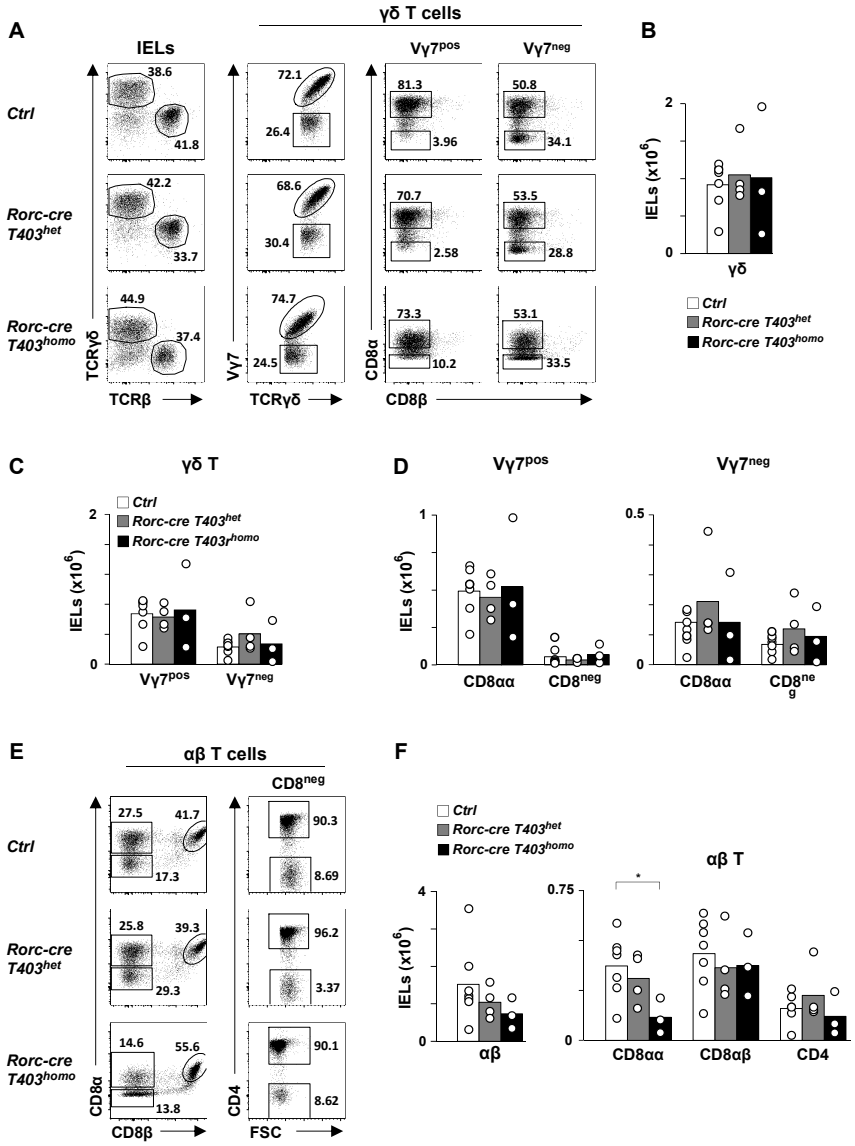

Figure 12 – RA imprints gut-tropism on CD8αα αβ T IELs after transition to DP stage in a dose-dependent manner. IELs were obtained from the guts of 8 weeks old *Rorc-cre T403*^{homo}, *Rorc-cre T403*^{het}, and WT litter-mate control mice. **A.** Representative FACS plots for total IELs and γδ T IEL subsets. **B.** γδ IELs cell numbers **C.** Vγ7-expressing IELs cell numbers. **D.** γδ IEL subsets cell numbers **E.** Representative FACS plots for αβ IEL subsets. **F.**αβ IELs cell numbers. Ctrl n=7; *Rorc-cre T403*^{het} n=4; *Rorc-cre T403*^{homo} n=3. *p<0.05, **p<0.01, and ***p<0.001.

As expected, both γδ T IELs and γδ T LPLs were not affected in *Rorc-cre T403* mice (Figures 12A, B, C and D and Figures 13A, B and C). Strinkingly, while unconventional CD8αα αβ IELs where reduced in *Rorc-cre T403*^{homo} mice, conventional αβ IELs (Figure 12E and F). Similarly, despite the profound decrease of *CD2-cre T403* αβ T LPL cell numbers, αβ T cells were not affected in the *lamina propria* of *Rorc-cre T403*^{homo} mice (Figures 13 D and E). Nevertheless, CD8αα αβ LPLs also tend to decrease in *Rorc-cre T403*^{homo} mice, but not to a significant level in this sample size (Figures 13A, D and E).

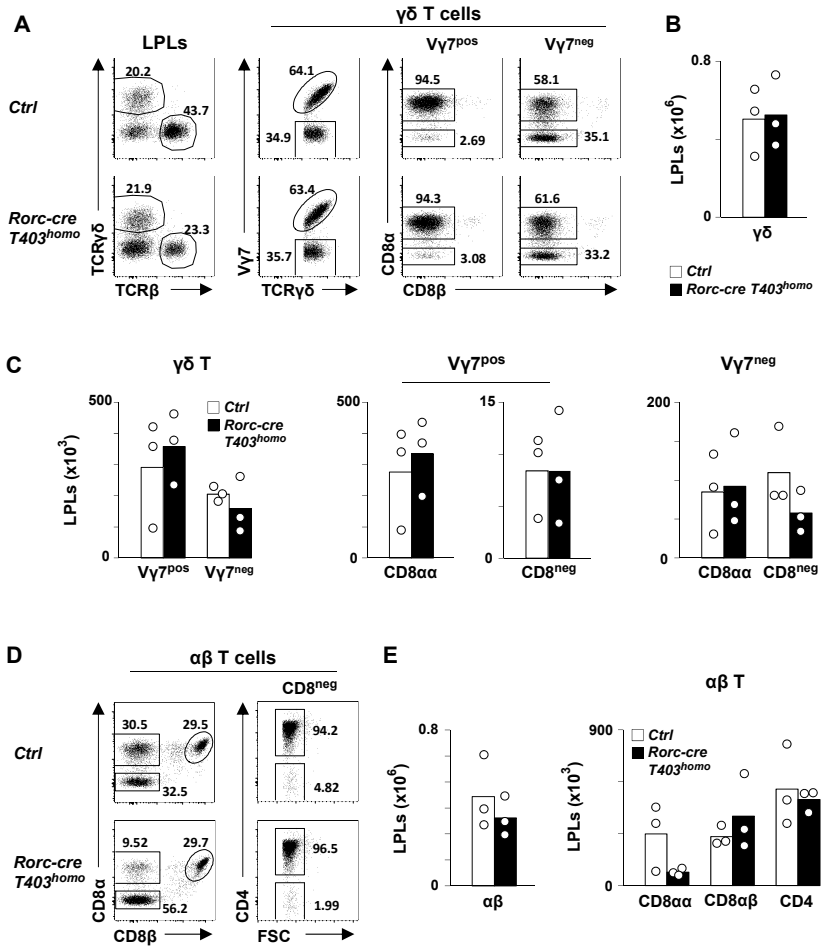

Figure 13 – RA did not substantially affect αβ T LPL numbers on *Rorc-cre T403*^{homo} mice. *Lamina propria* lymphocytes were obtained from the guts of 8 weeks old from the guts of 8 weeks old *Rorc-cre T403*^{homo}, and WT litter-mate control mice. **A.** Representative FACS plots for total LPLs and γδ T LPL subsets. **B.** γδ LPLs cell numbers **C.** γδ LPL Vγ7-expression and LPL subsets cell numbers **D.** Representative FACS plots for αβ LPL subsets. **E.** αβ LPL subsets cell numbers. Ctrl n=3; *Rorc-creT403*^{homo} n=3.

Taken together, our results suggest that conventional T cells require RA signalling before transition to the DP stage, since they were decreased in the gut of *CD2cre T403* mice but not in *Rorc-cre T403* mice. Additionally, these results also suggest that CD8αα αβ T cells require RA signals after transition to DP stage, notwithstanding that they may also require it before DP stage.

4. RA SIGNALLING CONTROLS IL-17 PRODUCTION IN UNCONVENTIONAL IELs

It was previously shown that RA regulates the production of IFNγ and IL-17 in enteric CD4 T cells, while suppressing the production of IL-17 in splenic CD8 T cells [135]. Similarly, RA enhances IFNγ and IL-22 production, but inhibits IL-17 production in LN γδ T cells [62]. Nonetheless, the role of RA signalling in γδ T IEL function remains elusive. Since IELs were highly impaired in the *CD2cre T403* intestines, we analysed if their function was also affected by RA signalling abrogation. *CD2cre T403^{het}* CD4 IELs had reduced IFNγ expression (Figures 14A and C). Strikingly, both *CD2cre T403^{het}* γδ T IELs and CD8αα^{pos} αβ T IELs displayed aberrant effector functions, with notably increased IL-17 production (Figures 14A, B and C). Thus, our data suggests that cell-autonomous RA signalling might control IL-17 production by γδ T IELs and CD8αα^{pos} αβ T IELs.

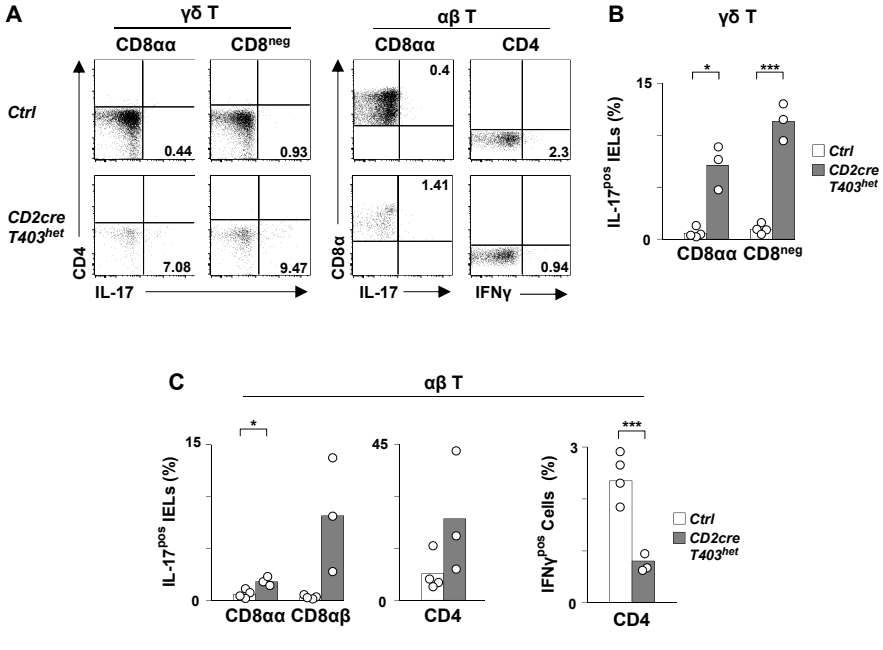

Figure 14 – Cell-autonomous RA signalling controls IL-17 expression by γδ IELs and CD8αα αβ T IELs. Enteric Intraepithelial lymphocytes were obtained from 8weeks old *CD2creT403^{het}* and their WT litter-mate controls. **A.** Representative FACS plots for the cytokine production of IEL subsets. **B.** Percentages of IL-17-producing γδ IEL subsets. **C.** Percentages of IL17-expressing αβ T IEL subsets and IFNγ-expressing CD4 αβ T IELs. Ctrl n=4; *CD2creT403^{het}* n=3. *p<0.05, **p<0.01, and ***p<0.001.

5. RA SIGNALLING CONTROLS ENTERIC ILC3s

Group 3 Innate Lymphoid Cells (ILC3s) are major innate components of the intestinal immune system. Previous work in our laboratory showed that cell-autonomous RA signalling was of paramount importance to the development of a foetal ILC3 subset, lymphoid tissue inducer cells (LTis). Thus, we aimed at determining if RA signalling is required for ILC3 development and function in adulthood.

Cre expression under *CD2* control genetically marks approximately half the bone marrow-derived Common Lymphoid Progenitors (CLPs) and 50% of enteric ILC3s (Annex 1 – Figure S1A and B). Taking this into consideration, RA signalling blockade should occur before ILC development, although it will only affect half of the ILC3s. Whilst *CD2cre T403^{het}* ILC3 cell numbers were comparable, *CD2cre T403^{homo}* ILC3s were heavily reduced (Figures 15A and D). In percentage, *CD2cre T403^{homo}* ILC3s were 1.7 fold decreased comparatively to WT counterparts (Figures 15A and B). This decrease was more pronounced in NCR^{neg} ILC3s (Figures 15A and D). Taken this, our results suggest that the enteric ILC3 pool may rely on RA signalling events.

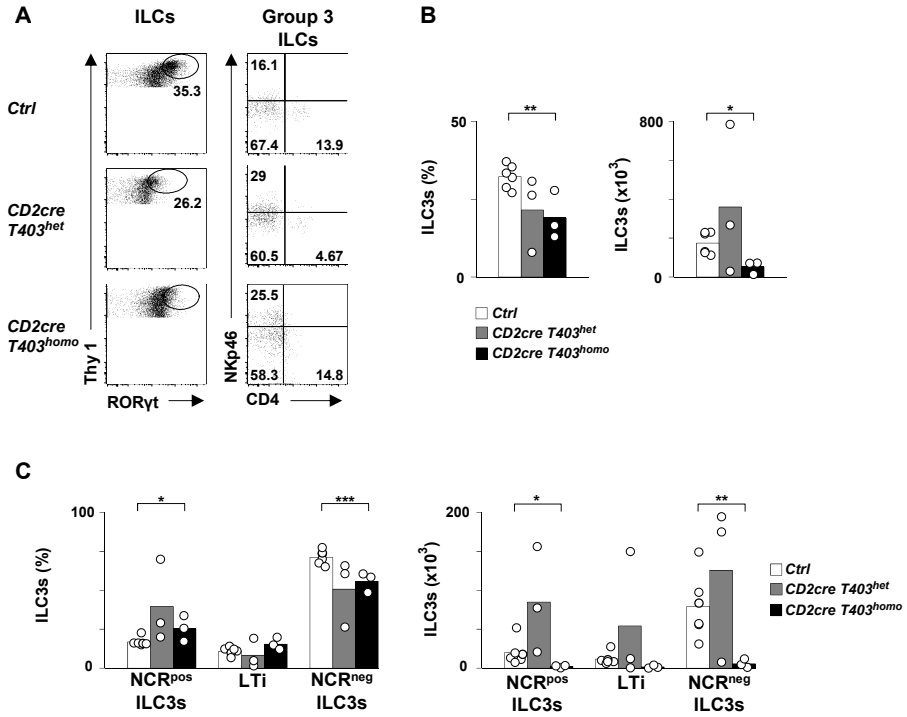

Figure 15 –RA signalling controls ILC3s in a dose-dependent manner. ILCs were obtained from the guts of 8 weeks old *CD2creT403^{homo}*, *CD2creT403^{het}* and their WT litter-mate controls. **A.** Representative FACS analysis plots for ILCs (identified as CD45^{pos}Thy1^{pos}Lin^{neg}; Lin markers : Gr-1, TER-119, CD11c, CD3, CD19, TCRβ, TCRδ, CD8α and NK1.1^{high}). **B.** ILC3s percentages and numbers. **C.** ILC3 subsets percentages and numbers. Ctrl n=6; *CD2creT403^{het}* n=3; *CD2creT403^{homo}* =3. *p<0.05, **p<0.01, and ***p<0.001.

The expansion of intestinal ILC pool not only occurs by newly generated ILCs, but also by proliferation of already existing ones [106]. In order to evaluate if *CD2cre T403^{homo}* ILCs reduction

was caused by impairment of proliferative capacity or due to increased cell-death, we evaluated the incorporation of BrdU and the staining for annexin V respectively. Interestingly, *CD2cre T403^{homo}* and WT ILC3 proliferative profile and apoptotic cell staining were similar (Figure 16).

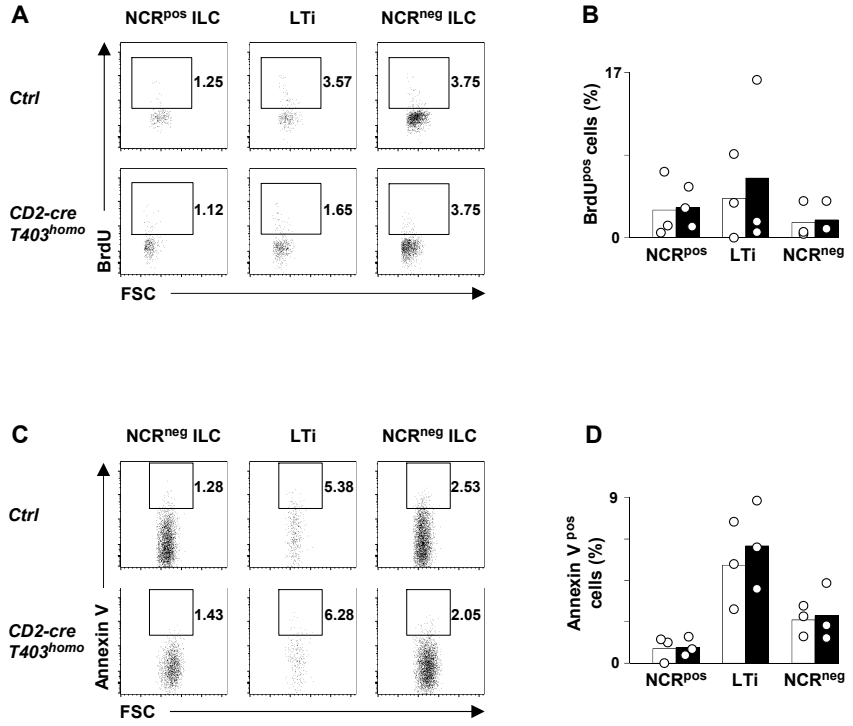

Figure 16 – RA signalling does not control cell-cycle and cell-death of ILCs. Mice were injected i.p. with BrdU 36h and 12h before analysis, or stained for annexin V. **A.** Representative FACS analysis plots for BrdU^{pos} ILC3 subsets. **B.** Percentage of BrdU^{pos} ILC3 subsets. **C.** Representative FACS analysis for annexin V staining of ILC3s subsets. **D.** Percentage of LC3s positive for annexin V staining. Ctrl n=3; *CD2creT403^{homo}* =3.

The reduction of *CD2cre T403^{homo}* ILC3s led us to investigate whether RA controlled ILC3s later in development, after ILC3-lineage commitment. ILC3s are all YFP^{pos} on *Rorc-cre* reporter mice (Annex 1 – Figure S1B), making this mouse model more suitable for ILC3 studies. Thus, we used *Rorc-cre T403* mice to achieve *Cre* recombination after ILC3 lineage-commitment, which is determined on the acquisition of RORγt [17]. *Rorc-cre T403^{homo}* ILC3s and particularly NCR^{neg} ILC3 pool were substantially reduced (Figures 17A, B and D). In addition, medium fluorescence intensity (MFI) of RORγt in *Rorc-cre T403^{homo}* ILC3s was greatly decreased (Figure 17C), reflecting decreased protein levels of this transcription factor. Altogether, our data suggests that RA signalling is fundamental for enteric ILC3 differentiation.

Furthermore, we explored if the down-regulation of genes critical for ILCs maintenance could justify the decreased ILCs numbers found in the absence of RA signalling. The expression of *Tox*, *Id2* and *Ahr* in ILC3s – pivotal for ILCs development – was comparable in *Rorc-cre T403^{het}* mice and their control counterparts (Figure 18). Interestingly, the expression of anti-apoptotic genes *Bcl2* and *Bcl2l1* was not altered in *Rorc-cre T403^{het}* ILC3 subsets, suggesting that RA may not impact ILCs survival. Moreover, transcript levels of *Il-23r* – a receptor that engages IL-

23 and elicits the production of IL-22 on ILC3s – were reduced in *Rorc-cre T403*^{het} LTis (Figure 18). Importantly, we found a profound down-regulation of *Rorc* –fundamental for ILCs differentiation – in *Rorc-cre T403*^{het} NCR^{pos} ILC3s (Figure 18).

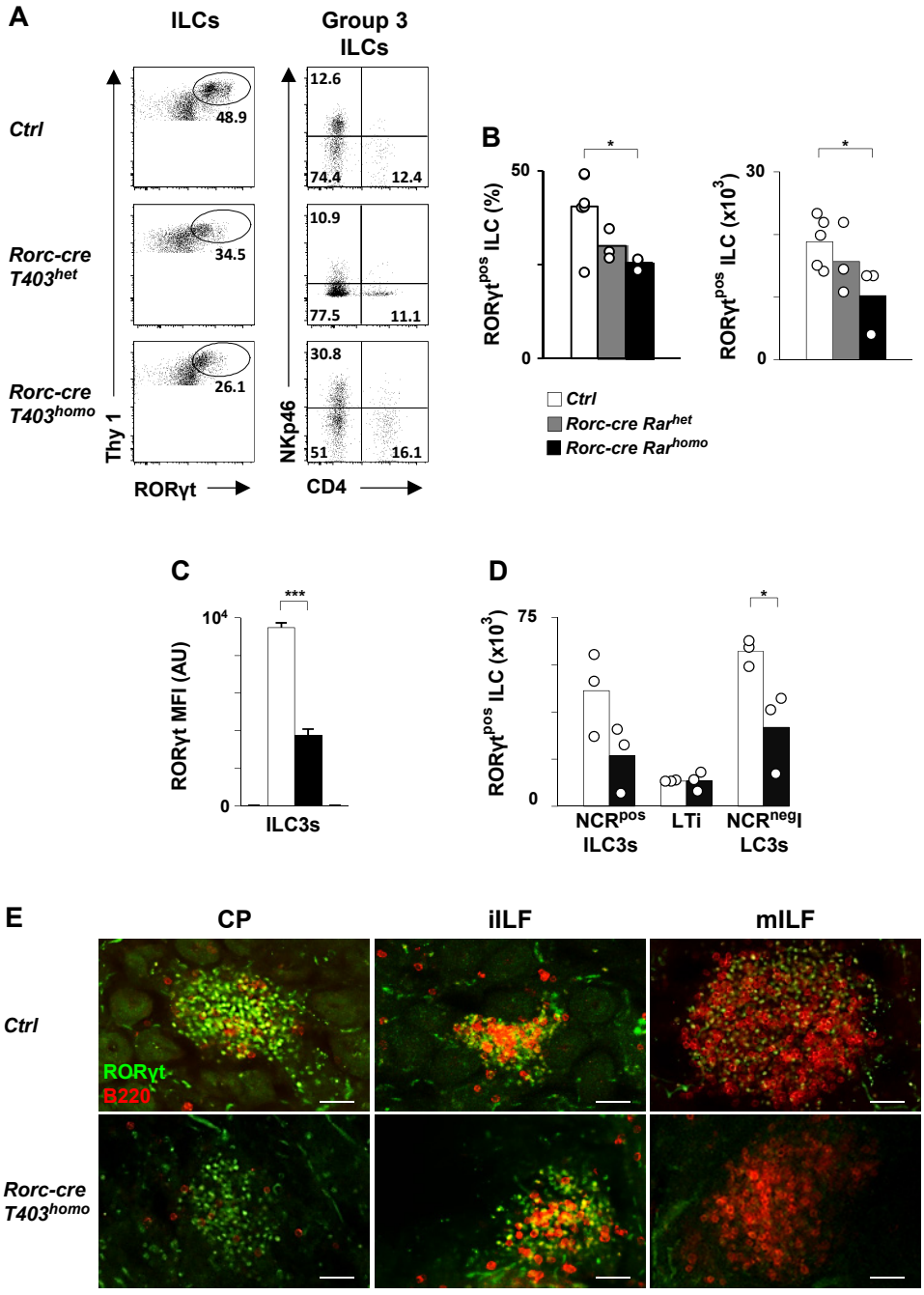

Figure 17 – Cell-autonomous RA signalling controls ILC3s in a dose-dependent manner. *Lamina propria* cells were obtained from the guts of 8 weeks old *Rorc-cre T403*^{het} *Rorc-cre T403*^{homo} and WT litter-mate control mice. **A.** Representative FACS analysis plots for ILC3s. **B.** ILC3s percentages and cell numbers. **C.** Medium intensity fluorescence (MFI) of RORγt in ILC3 cells. **D.** ILC3 subsets percentages and cell numbers. Ctrl n=6; *Rorc-cre T403*^{het} n=3; *Rorc-cre T403*^{homo} n=3. **E.** Whole mount confocal imaging of 8 weeks old *Rorc-cre T403*^{homo} and WT litter-mate control mice intestines. ILCs were stained with anti-RORγt and B cells with anti-B220. Error bars correspond to the standard error of the mean (SEM). Ctrl n=1; *Rorc-cre T403*^{homo} n=1; Scale bars: 20; Original magnification: ×20. *p<0.05, **p<0.01, and ***p<0.001.

Finally, given the profound reduction in *Rorc-cre T403^{homo}* ILCs we assessed whether RA signalling was essential for ILC3 clustering into CPs and ILFs. Confocal microscopy analysis of *Rorc-cre T403^{homo}* 8 week-olds revealed normal CP and ILF development (Figure 17E).

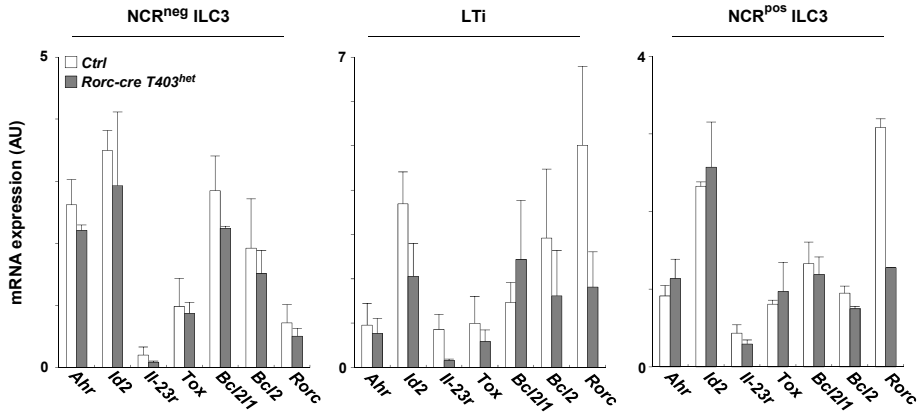

Figure 18 – RA regulates *Rorc* expression in NCR^{pos} ILCs. ILC3s were isolated from the guts of 8 weeks old *Rorc-cre T403^{het}* and WT litter-mate control mice. **A.** mRNA expression of *Rorc-cre T403^{het}* ILC subsets. Ctrl n=2; *Rorc-cre T403^{het}* n=2.

Cumulatively, our data suggest that RA controls ILCs numbers in a dose-dependent manner. Our results also indicate that the reduction of *Rorc-cre T403* ILC3s may not be caused by a proliferation and survival deficit, but rather by an impairment of their differentiation. In addition, we suggest a putative role of RA signalling in the maintenance of RORγt expression on ILC3s.

VII. DISCUSSION

Higher organisms are continuously exposed to a myriad of foreign components and microorganisms that may threaten their normal function. Mucosal tissues are the main route of microbial invasion, requiring a specialized immune system than maintains its integrity and enables effective protection against these insults. The enteric immune system harbours diffuse lymphocytes that reside in the *lamina propria* and T cells embedded in the basolateral sides of epithelial cells (ECs). The latter, called intraepithelial lymphocytes (IELs), are key components of the first-line defence against luminal pathogens [2]. The intestinal *lamina propria* harbours innate lymphoid cells (ILCs) which are major innate components of the enteric immune system [68, 108]. However, the factors regulating ILCs and IELs in the intestine are still very elusive. Retinoic acid (RA), which is a diet-derived nutrient has been tightly associated with numerous properties that range from SLO organogenesis to the modulation of T cell responses in the periphery[17, 123]. Yet, how RA cues contribute for IEL and ILC development, migration and function has not been unveiled. Herein, we disclose the role of cell-autonomous RA signalling in the regulation of enteric immune system, particularly in the IEL compartment.

In the present work, we reveal that signal events elicited by RA are of paramount importance for the regulation of enteric T cells, in particular IELs. Primarily, we found that abrogation of RA signalling in all T cells resulted in profoundly reduced numbers of γδ T, CD8αα and CD8αβ αβ T IELs. As previously described with vitamin A deficient diets,[84] a similar phenotype was present on the *lamina propria* lymphocyte (LPL) compartment, as all *CD2cre T403^{het}* LPLs were heavily decreased.

Since gut-colonization of T cells mainly occurs during the first 3 weeks of life, we analysed if a reduction of *CD2cre T403* enteric T cells was already present at this stage. Indeed, the evaluation of thymic cells revealed that *CD2cre T403^{het}* unconventional T cells were decreased in the gut, whilst they were increased in the thymus. In addition, *CD2cre T403^{het}* conventional CD8αβ αβ T cells were also increased in the thymus, although they were comparable in the gut. Since there is increasing data consistent with the hypothesis of T cells being automatically directed from the thymus to the intestine,[2, 69, 82, 86, 87, 88] our results suggest that unconventional T cells and conventional CD8αβ αβ T cells tropism is imprinted by RA in the thymus. However, in order to ascertain if there is a thymic-imprinting of gut-tropism during gut-colonization, in future work we intend to evaluate the expression of gut-homing molecules at this developmental time-point. In addition, since T cells were increased in the thymus at this developmental stage, we

believe that IEL reduction is not caused by an impairment of T cell development during gut colonization.

Our results suggest that unconventional T cell gut-homing may rely on RA signalling. Accordingly, gene expression of *Ccr9* – indispensable for gut-residency [83] – was profoundly down-regulated in all *CD2cre T403*^{het} IEL subsets. However, not only is CCR9 required for gut-homing of T cells, but it is essential for thymic-ingress of T cell progenitors as well.[140] As CCR9 expression is regulated by RA[84], and since *CD2cre* has a penetrance of approximately half the CLPs (Annex 1 – Figure S1) and virtually all DN cells[136], this raises the question whether RA influences the arrival of progenitor cells to the thymus or even affects T cell-development in adulthood. In order to answer this question, we intend to analyse the expression of CCR9 in the thymus and the numbers of T cell progenitors of *CD2cre T403* mice. In order to assess if thymic T cell development is controlled by RA, in the future we intend to perform RTOCs (Reaggregation thymus organ cultures) that enable T cell reconstitution in the presence of RA or BMS, an antagonist of RARs.

Since CCR9 expression was down-regulated in almost all *CD2cre T403*^{het} T cell subsets, we followed the hypothesis that an impairment of gut-homing was the most probable cause of IEL reduction. Support to this hypothesis was provided by the analysis of IELs at the molecular level. Not only *Ccr9* was down-regulated in all IELs, but also *Itga4* expression, which is critical for T cell homing to the intestine, was reduced in *CD2cre T403*^{het} γδ T cells. On the other hand, the increased *Itgae* and *Itgb7* expression found on *CD2cre T403*^{het} CD8αα IELs may reflect the fact that only thymocytes with increased $\alpha_E\beta_7$ expression are able to successfully populate the intestinal epithelium in the absence of retinoid signalling. However, as we did not evaluate the expression of α_E in *CD2cre T403* thymocytes, we cannot assess if this increased expression is imprinted in the thymus. If so, since α_E is also present in thymocytes and binds strongly to thymic epithelial cells[142], T cells can also be arrested in the thymus. In other words, RA signal events may not only impact the ability of T cells to reside in the gut, but also their egress from the thymus. In order to answer this question, we plan to monitor thymocytes by injecting FITC intrathymically and tracing FITC^{pos} T cells in circulation. In addition, we also intend to analyse the expression of molecules that are indispensable for thymic-egress, such as S1P1 in *CD2cre T403* thymocytes.

Our results also suggest that cell-autonomous RA signalling does not affect T cell survival and expansion. Indeed, levels of *Ahr*, required for the maintenance of IELs, were similar in the absence or presence of RA signalling. Importantly, as the proportion of BrdU^{pos} IELs did not

alter in *CD2cre T403* mice, we considered that the ablation of RA signalling did not influence IEL proliferative capacity and expansion. Moreover, the up-regulation of anti-apoptotic genes *Bcl2* and *Bcl2l1* on *CD2cre T403^{het}* IELs does not favour the hypothesis of an increased T cell-death in the absence of RA signalling. However, since multiple proteins are involved in apoptosis, *Bcl2* and *Bcl2l1* expression is only allusive, being insufficient to evaluate cell-survival. As we did not evaluate cell-death of IELs directly, we intend to stain *CD2cre T403* IELs with annexin V in order to assess the propensity to apoptosis in the abrogation of RA signalling.

Our findings suggest that RA imprinting of gut-homing and/or thymic egress on T cells may occur at different developmental stages. In agreement to this, we found that whereas nearly all *CD2cre T403^{het}* and *CD2cre T403^{homo}* IELs were decreased, only CD8αα αβ T IELs were affected in *Rorc-cre T403^{homo}* mice. Therefore, as *Rorc* expression is firstly expressed during T cell development at the DP stage [21, 138], our results suggest that RA imprints gut-tropism on CD8αα αβ T IELs after the transition to DP stage, where CD8αα lineage commitment occurs. Since conventional *Rorc-cre* CD8αβ αβ T IELs were not impaired, they may require RA-induced gut-tropism before this transition. Nevertheless, we could not assess the developmental stage at which γδ T cells may be gut-imprinted since they derive from DN2 and DN3 cells, and therefore do not undergo DP stage. The absence of a specific marker for γδ T cells impedes the development of lineage-specific *Cre* mice in order to answer this question.

In addition, our data suggest that RA may be a key regulator of unconventional IEL responses. First, in agreement to studies highlighting RA promotion of IFNγ responses on CD4 T cells[135], CD4 IELs revealed decreased IFNγ production in *CD2cre T403^{het}* mice. On the other hand, *CD2cre T403^{het}* γδ T cells and CD8αα αβ T IELs displayed aberrant IL-17 production. However, since *CD2cre T403^{het}* enteric T cells were highly diminished, the few existing unconventional IELs could be deregulated, therefore increasing IL-17 production in a non cell-autonomous manner. We plan to address this question by developing lymphocyte-replete chimeras displaying WT T cells and *CD2cre T403^{het}* T cells, distinguishable by the expression of different congenic markers. It would be also interesting to evaluate the immune responses of IELs during enteric infection, in the absence of optimal RA signals. In the near future we plan to examine IEL responses in these chimeras, following oral infection with a *Listeria monocytogenes* (*Lm*) strain that invades epithelial cells in mice [90].

In this work we also found that RA critically impacts Group 3 ILCs cell numbers in a dose-dependent manner, as *CD2cre T403^{homo}* ILC3s but not *CD2cre T403^{het}* ILC3s were severely reduced. Concomitantly with *CD2cre T403^{homo}* T cells depletion, *CD2cre T403^{homo}* ILC3s were

decreased by half in percentage, correlating with the penetrance of *Cre* expression under *Cd2* control on ILC3s. Our data also suggest that cell-autonomous RA signalling events may be pivotal for the effectiveness of ILC3 differentiation. In agreement, *Rorc* expression – which is fundamental for ILC3 differentiation – was down-regulated in *Rorc-cre T403^{het}* NCR^{pos} ILC3s and LTi cells. In addition, *Rorc-cre T403^{homo}* ILC3 numbers were substantially reduced, particularly NCR^{neg} ILC3s. As ILCs proliferate in order to expand [106], a decrease in ILC pool could correlate with a defective proliferative capacity. However, and further evincing RA function on ILC3 differentiation, BrdU incorporation was similar between *CD2cre T403^{homo}* and their WT counterparts. Similarly, a decrease resistance to apoptosis could be affecting ILCs survival. However, *Rorc-cre T403^{het}* ILC3s displayed comparable expression of *Bcl2* and *Bcl2l1* anti-apoptotic partners suggesting that RA may be dispensable for ILCs survival. In fact, Annexin V assays revealed similar percentage of apoptotic ILC3s between *CD2cre T403^{homo}* and WT mice, favouring the hypothesis that increased cell-death is not the cause of ILCs depletion in mice with deficient RA signalling.

Remarkably, our results suggest that ILCs reduction in mice with deficient RA signalling does not affect ILCs assembly into cryptopatches (CP) and CP maturation into ILFs. We observed that *Rorc-cre T403^{homo}* ILC3s still form cryptopatches and ILFs normal in size and morphology. However, since we did not count the number of CPs and ILFs we cannot assess if the reduction of ILCs resulted in decreased number of these structures. In the future, we plan to count the number of CPs and ILFs in *Rorc-cre T403^{homo}* intestines in order to assess with more certainty if CPs and ILFs formation is affected, in steady-state and in inflammation following dextran sodium sulphate (DSS) -induced colitis. In future work we also plan to assess ILCs function in the absence of optimal RA signalling. To do so, we intend to analyse the cytokine profiles of *Rorc-cre T403^{homo}* enteric ILC3s. We will also infect *Rorc-cre T403^{homo}* mice in *Rag2*$^{neg/neg}$ background with *Citrobacter rodentium.* With this, we aim the assessment of cell-autonomous RA role in ILC3 IL-22 production during stress-causing events. Additionally, we also intend to treat *Rorc-cre T403^{homo}* mice with DSS in order to study the relevance of RA signalling in ILCs function during chronic inflammation.

In summary, retinoic acid, a nutrient that has pleiotropic effects in foetal development, rises as a factor that critically controls enteric IELs, and also regulates ILCs. Notably, RA cues seem able to prime T cells with tropism to the intestinal epithelium. Although peripheral dendritic cells (DCs) have been reported as the main inducers of CCR9 expression in T cells, we believe that T cells may also acquire this marker in the thymus via RA signals. Not only RA seems able to control T cells in the intestinal epithelium, but also appears to regulate IEL responses,

particularly IL-17 production. Additionally, we propose that ILCs differentiation is controlled by RA signalling. Collectively, our results argue that diet-derived nutrients are key coordinators of the intestinal immune system.

VIII. ANNEXES

ANNEX 1 – SUPPLEMENTARY FIGURES

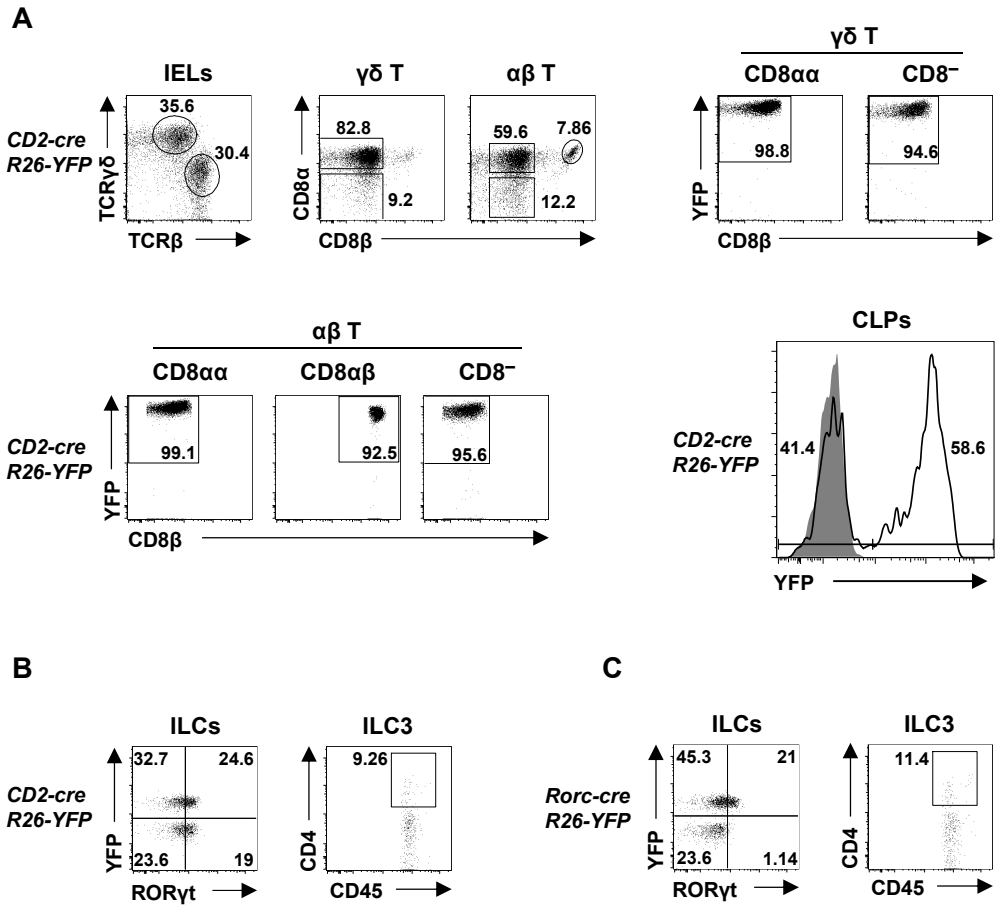

Figure S1 – *Cre* efficiency under *Cd2* and *Rorc* control in IELs. IEL and LPLs were extracted from the guts and CLPs were obtained from the femur bone marrow of adult *CD2cre R26-YFP* reporter mice. ILCs were prepared from *Rorc-cre R26-YFP* reporter mice *lamina propria*. Cell suspensions were then properly stained for their particular populations and assessed by flow citometry. Expression of *Cre* in **A.** IELs and CLPs from *CD2cre R26-YFP* mice **B**. ILCs from *CD2cre R26-YFP* mice.**C.** ILCs from *Rorc-cre R26-YFP* mice. *CD2cre R26-YFP* mice n=1; *Rorc-cre R26-YFP* n=1

ANNEX 2 – USED SOLUTIONS

- **PBS 1X:** 10% phosphate buffer saline (PBS) 10X (GIBCO) in MilliQ water.

- **Complete medium:** RPMI medium with 10% fetal bovine serum (FBS), 10.000 U Streptomycin and Penicillin, 5% Glutamine, 5% HEPES buffer, 5% Sodium Pyruvate and 500µL β-mercaptoethanol from GIBCO.

- **IEL isolation medium:** complete medium with 1mM ditiotreitol (DTT) (Sigma).

- **Digestion medium:** complete medium with 5mg/ml Collagenase D and 1mg/ml DNase I (Roche).

- **Activation medium:** complete medium with Brefeldin A, PMA and Ionomycin

- **FACS BUFFER:** PBS 1X with 2% FBS.

- **1X Perm Buffer:** 10% Perm Buffer 10X (eBiosciences) in MilliQ water.

- **Blocking buffer:** 50mM Tris buffer pH 7.2, 150mM NaCl, 0.6%Triton X-100, 0.1% Bovine serum albumin (BSA)

- **Tail lysis Buffer:** 10mM Tris pH 8, 100mM NaCl, 10mM EDTA pH 8, 0.5% SDS in MilliQ water.

- **BABB:** solution of 2:1 of Benzyl Alcohol and Benzyl Benzoate

- **4% PFA:** 4g of paraformaldehyde (PFA) (Sigma) in 100 mL of blocking buffer

ANNEX 3 (A) – USED PRIMERS

Genotyping primers:

- ROSA-26 WT Allele

  Common R26 YFP: 5’ – AAG TCG CTC TGA GTT GTT AT– 3’
  WT R26 YFP: 5’ – GCG AAG AGT TTG TCC TCA ACC – 3’

- RARaT403 WT Allele

  RARA403 F: 5’ – ATGGTGTACACGTGTCACC – 3’
  RARA403 R: 5’ – CACCTTCTCAATGAGCTCC – 3’

- CD2-icre Allele

  CD2-iCre F: 5’– AGATGCCAGGACATCAGGAACCTG – 3'
  CD2-iCre R: 5'– ATCAGCCACACCAGACACAGAGATC – 3'

- Rorc-cre Allele

  Rorc-cre F: 5’– TTC CCG CAG AAC CTG AAG ATG TTC G – 3'
  Rorc-cre R: 5'– GCC AGA TTA CGT ATA TCC TGG CAG C – 3'

ANNEX 3 (B) – USED PROBES

qPCR probes:

Gene	Invitrogen Reference
Ltb	Mm00434774_g1
Itgb7	Mm01296188_m1
Itgae	Mm00434443_m1
Itga4	Mm01277951_m1
Ccr9	Mm02620030_s1
Bcl2	Mm00477631_m1
Bcl2l1	Mm00437783_m1
Ahr	Mm00478932_m1
Id2	Mm00711781_m1
Rorc	Mm01261022_m1
Tbx21	Mm00450960_m1
Tox	Mm00455231_m1
Il23r	Mm00519943_m1

ANNEX 4 – USED ANTIBODY MIXES

IELs mix: anti-Vγ7, anti-CD8b, anti-CD8a, anti-CD4, anti-TCRb, anti-TCRγδ, anti-CD45

LPLs mix: anti-CD8b, anti-CD8a, anti-CD4, anti-TCRb, anti-TCRγδ, anti-CD45

ILCs mix: anti-NKp46, anti-RORγt, anti-CD4, anti-Thy1.2, anti-CD45, anti-CD8a, anti-TCRb, anti-TCRγδ, anti-B220, anti-CD19, anti-CD3, anti-Gr-1, anti-CD11c, anti-TER119. Underlined antibodies are defined as 'lineage' (Lin) in ILCs FACS plots.

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